

# **Genetic Transformation, Regeneration and Analysis of Transgenic Peanut**

**Colleen M. Higgins and Ralf G. Dietzgen**

Queensland Government Department of Primary Industries  
Agency for Food and Fibre Sciences  
Queensland Agricultural Biotechnology Centre  
Gehrman Laboratories  
The University of Queensland  
St. Lucia, Qld 4072  
Australia

**ACIAR Technical Reports Series 48**

The Australian Centre for International Agricultural Research (ACIAR) was established in June 1982 by an Act of the Australian Parliament. Its mandate is to help identify agricultural problems in developing countries and to commission collaborative research between Australian and developing country researchers in fields where Australia has a special research competence.

Where trade names are used this constitutes neither endorsement of nor discrimination against any product by the Centre.

#### **ACIAR TECHNICAL REPORT SERIES**

This series of publications contains technical information resulting from ACIAR-supported programs, projects and workshops (for which proceedings are not published), reports on Centre-supported fact-finding studies, or reports on other useful topics resulting from ACIAR activities. Publications in the series are distributed internationally to selected individuals and scientific institutions.

© Australian Centre for International Agricultural Research,  
GPO Box 1571, Canberra, ACT., 2601 Australia

Colleen M. Higgins and Ralf G. Dietzgen 2000.  
Genetic Transformation, Regeneration and Analysis of Transgenic Peanut.  
ACIAR Technical Report Project No. 48. 86 pp.

ISBN 1 86320 276 5

Editorial management: P.W. Lynch

Production editing: PK Editorial Services Pty Ltd, Brisbane

Cover design: P.R. Campbell

Typesetting, page layout and illustrations: Sun Photoset Pty Ltd, Brisbane

Printing: Brown Prior Anderson, Melbourne

# CONTENTS

	Page
<b>Acknowledgments</b>	iv
<b>CHAPTER 1</b>	
<b>Introduction</b>	1
1.1 Improved diagnosis and control of peanut stripe virus	1
1.2 Genetic transformation of peanut	1
<b>CHAPTER 2</b>	
<b>Genetic Transformation of Peanut</b>	5
2.1 Peanut transformation via microprojectile bombardment	5
2.2 <i>Agrobacterium tumefaciens</i> -mediated transformation of peanut	22
(A) Transformation using leaflets as explants and regeneration via organogenesis	23
(B) Transformation using somatic embryos as explants, regeneration via embryogenesis and organogenesis	34
2.3 Recipes	37
<b>CHAPTER 3</b>	
<b>Analysis of Transgenic Peanut Lines</b>	43
3.1 Initial screen for transgenic tissues: PCR	43
3.2 Transgene integration: Southern blot	46
3.3 Transgene expression: mRNA	61
3.4 Transgene expression: protein	67
3.5 Recipes	74
<b>References</b>	84

## **ACKNOWLEDGMENTS**

The authors thank the following persons involved with ACIAR project 9439 for their contributions during the project and/or assistance in preparation of this Technical Report:

At the Queensland Department of Primary Industries and the University of Queensland, Dr Robert G. Birch, Dr D. Malcolm Livingstone, Ms Rhonda M. Hall, Mr Phil LaBrie, Dr Neena Mitter, Ms Tanya Newton, Ms Seetha Karunaratne, Dr Shiromani Basnayake, Ms Lyndal Hill, Ms Berlinda Heilersig, Mr Jonathan Anderson, Mr Alan Cruickshank and Mr Jeff Tatnell;

At the Institut Pertanian Bogor, Indonesia, Dr Sudarsono and Mr Soleh Avivi;

At the Institute for Oil Crops Research, Wuhan, China, Prof. Xu Zeyong, Mr Chen Kunrong, Mr Fang Xiaoping and Ms Yan Li Ling.

## **DEDICATION**

This report is dedicated to the late Keith Middleton whose insight into international peanut production and vision for the applications of biotechnology started this research.

# CHAPTER 1

## Introduction

### 1.1 Improved diagnosis and control of peanut stripe virus

ACIAR projects 8419 (1985–1988) and 8834 (1989–1991) identified peanut stripe virus (PStV) among the major constraints to peanut production in Indonesia. No sources of resistance to this virus were found in more than 10 000 accessions of the world *Arachis hypogaea* germplasm collection in collaboration with ICRISAT. Classical breeding approaches incorporating host resistance genes from wild *Arachis* relatives proved unsuccessful due to genetic incompatibility.

Therefore, two subsequent ACIAR projects, 9017 (1992–1995) and 9439 (1996–1999) addressed the provision of protection against PStV in commercial peanut lines by incorporation of transgenic resistance genes derived from the virus itself. A prerequisite to the introduction and expression or silencing of transgenes is a reliable and effective means of introducing novel genes into peanut tissue and subsequent regeneration of transformed plants which carry and stably inherit the novel resistance trait.

During projects 9017 and 9439, several approaches to gene delivery, types of peanut explants and different avenues for regeneration of plantlets were attempted which have been summarised in the projects' Annual Reports and in Livingstone and Birch (1995, 1999). The development of the detailed peanut transformation protocols described here formed the basis of BSc Honours thesis for D. Malcolm Livingstone and Tanya Newton and D.M. Livingstone's PhD thesis (Botany Department, The University of Queensland).

### 1.2 Genetic transformation of peanut

#### Why transform peanut?

Conventional breeding has led to the improvement of some peanut traits such as seed yield and drought tolerance. However, many important agronomic traits have yet to be improved. These include resistance to insect pests as well as to diseases caused by nematodes, viruses such as *Tomato spotted wilt* and peanut stripe viruses, bacteria, leaf spot fungi and to fungi such as *Aspergillus flavus* and *A. parasiticus* which produce carcinogenic aflatoxin (Dietzgen 1999). Other beneficial traits would include tolerance to water stress, uniform fruit maturity, enhanced nutritional quality by modification of the amino acid and lipid composition.

It is believed that a single hybridisation event between *Arachis duranensis* and *A. ipaensis*, both diploid species, gave rise to what we recognise as peanut today. Thus, peanut has a very narrow genetic base (Kochert 1996). There is little in the way of resistance genes to fungal or viral diseases available to breeders. Furthermore, introgression of desirable traits, if available, from wild *Arachis* relatives is difficult, especially as peanut is a self-pollinating species. Few lines derived from interspecific crosses have been made available to peanut growers. Lines showing resistance to *Cercospora arachidicola* and *Meloidogyne arenaria* have been released. However, these lines are low yielding with poor agronomic quality (Kokalis-Burelle et al. 1997). Thus, extensive peanut breeding programs are needed in addition to the interspecific crossing to impart this resistance to commercially viable lines.

Introduction of novel traits into peanut using genetic engineering techniques will assist in the development of improved peanuts by allowing the introduction of genes from sources other than *Arachis* germplasm directly into commercially important cultivars without altering other characteristics.

## Tissue culture and gene transfer

Successful genetic transformation of plants, including peanut, generally requires a tissue culture system to regenerate whole fertile plants from single cells as well as a method to deliver the gene(s) of interest to those cells that have the capacity to regenerate.

Regeneration of whole plants from explants such as protoplasts, cell suspension cultures, callus tissue or organised tissue such as embryonic axes has been reported for many species. Peanut plants have been regenerated from explants such as mature and immature embryonic axes (Baker et al. 1995; Brar et al. 1994; Hazra et al. 1989; Atreya et al. 1984), cotyledons (Ozias-Akins 1989; Atreya et al. 1984) and leaves (Livingstone and Birch 1995; Baker and Wetzstein 1992) by either organogenesis or embryogenesis. There has been one report of *Arachis paraguariensis* regeneration from protoplasts using a nurse cell culture method (Li et al. 1993) but this has not been successful for *A. hypogaea* cultivars. Generally, the published methods for peanut regeneration have been inefficient, inconsistent and genotype-dependent. In addition, few protocols have been reported for the regeneration of economically important commercial cultivars.

Two commonly used means of delivering genes to plant cells are via *Agrobacterium tumefaciens* or direct gene transfer using microprojectile bombardment. *A. tumefaciens* is a soil bacterium, which transfers specific DNA molecules into cells of wounded host plants, causing crown gall disease. *A. tumefaciens* carries a tumour inducing (Ti) plasmid which contains a region called the T-DNA that is transferred into a plant cell where it becomes integrated as part of the plant's genetic material (Chilton 1983). It has been possible to replace gall-inducing genes normally found within this T-DNA with genes of agricultural interest, such as disease resistance genes. When *A. tumefaciens* transfers these genes to plant cells at the wound site that are capable of regeneration, stably transformed plants carrying the new trait can be obtained.

Microprojectile bombardment involves the coating of gold or tungsten particles with DNA and accelerating them at high velocity into target plant tissue. Some particles will penetrate into the nuclei of some of the cells, where the DNA may integrate into the plant's genome. Cells that survive the impact and are able to regenerate can give rise to whole transgenic plants.

The choice between using microprojectile bombardment or *Agrobacterium* as the means by which to deliver DNA is determined by several factors including the laboratory facilities and technical skills available, the species and/or cultivar to be transformed (many monocots are still recalcitrant to transformation with *Agrobacterium*, although this is improving all the time), and the regeneration system. There is a general belief that *Agrobacterium*-mediated transformation is preferable to microprojectile bombardment for gene delivery. This belief is based on several assumptions, which are becoming less compelling due to recent new information. *Agrobacterium*-mediated transformation is thought to result in fewer integrated transgene copies, thus reducing the risk of transgene rearrangements and gene silencing.

Further, *Agrobacterium* is considered a 'cleaner' approach, since the T-DNA is thought to be the only DNA which is transferred. It is now known that DNA from outside the T-DNA region can also be transferred into the plant's genome (Smith 1998). Microprojectile bombardment can result in multiple transgene copies. However, we have observed in many cases that the copy number is equivalent to that observed using *Agrobacterium*. The transgene copy number and hence the chances of gene rearrangements can be reduced by careful optimisation of bombardment conditions. Thus, it is our opinion that microprojectile bombardment is an appropriate means by which to genetically modify important crop species, including peanut.

Uptake of DNA transferred by either method only occurs in a minority of cells. Inclusion of a gene encoding resistance to an antibiotic such as kanamycin or hygromycin, or to a herbicide such as phosphinothrinicin, in addition to the gene(s) of interest, allows the selection of such cells, if the compound is included in the nutrient medium. Cells that express the resistance gene can proliferate while the untransformed cells die. For peanut, hygromycin B is the most appropriate compound for the selection of transformed cells. Kanamycin has been shown to be an ineffective selective agent for peanut as for other legume species (Khan et al. 1994), since many 'escapes' (untransformed plants) regenerate in the presence of this antibiotic (Clemente et al. 1992; Newton 1997). The herbicide Basta® (active ingredient phosphinothrinicin) has also been used to select transgenic peanut tissue (Brar et al. 1994). However, we considered its use inappropriate for peanut, because Basta is used by growers in Australia to eliminate peanut volunteer plants after harvest and to exclude potential pollen spread of the herbicide resistance gene to other crops and weeds.

## Intended practical applications

The recent development of transformation and regeneration systems has allowed the introduction of useful genes into peanut germplasm. The understanding of disease resistance mechanisms is becoming increasingly advanced so that it should be possible to apply technologies that have been proven in other species to peanut. For example, it has been demonstrated that the transfer of viral coat protein gene sequences may confer specific resistance to that virus. This is the approach we have taken to develop commercially important peanut cultivars with resistance to peanut stripe virus. Yang et al. (1998) reported the transfer of the *Tomato spotted wilt virus* nucleocapsid gene as a means by which to protect peanut from infection by this virus. Urban and Weissinger (1996) reported their intention to use the osmotin and cecropin genes to confer fungus resistance to peanut cvs. Valencia A and NC7, respectively.

The practical application of novel genes in plants often requires tissue-specific expression rather than expression in all parts of the plant. The isolation of gene regulatory regions (promoters) is important for understanding the regulation of plant gene expression. Determining the activity of a regulatory sequence, i.e., where and when a gene is expressed as well as the level of expression, provides information as to how a transgene will be expressed in a transgenic plant when regulated by such a promoter sequence. Smith and Beliaev (1995) recently reported the isolation of peanut seed coat- and pod-specific genes as a means of isolating their promoters. Such promoters can then be used to express disease resistance genes specifically in the seed coat and pod. Ozias-Akins (1996) also reported the use of a

pod-specific promoter from soybean that could be used to express genes in peanut. This promoter has been used to express a *Bacillus thuringiensis* toxin gene to confer resistance to the lesser cornstalk borer.

In this Technical Report, we describe protocols for the routine regeneration of transformed peanut plants of spanish and virginia market types based on microprojectile bombardment of embryogenic callus as well as *Agrobacterium*-mediated transformation of somatic embryos. Whatever the transformation method to be adopted in the reader's laboratory, the application of scientific advances to the improvement of peanut will broaden its genetic base and will assist future peanut breeding programs and the peanut industry.

## CHAPTER 2

# Genetic Transformation of Peanut

### 2.1 Peanut transformation via microprojectile bombardment

THE use of microprojectile bombardment as a means of developing transformed peanut plants was first reported by Ozias-Akins et al. (1993) using the cultivar Toalson. This method was developed using immature peanut seeds as the source of explants. Bombardment of 1–2 year old embryogenic callus derived from immature embryos and cotyledons resulted in 1% of bombarded callus pieces producing stable, transformed lines. It was not reported if the plants were fertile. However, a more recent report indicated that the efficiency has been improved 25-fold and that plants are capable of producing pods (Ozias-Akins et al. 1996).

There have been other reports of microprojectile bombardment of peanut tissue. For example, Livingstone and Birch (1995) and Clemente et al. (1992) reported bombarding leaflets from mature embryos. However, neither group reported the regeneration of transformed plants using this target tissue. Brar et al. (1994), on the other hand, reported transformation and regeneration of fertile peanut plants using the ACCELL bombardment system on exposed meristems from mature seeds of the elite cultivars Florigiant and Florunner. Since transformed lines were identified via histochemical staining for GUS activity, this method would be likely to result in chimeric plants.

We have also been able to regenerate transformed peanut plants using immature embryos from several cultivars including cvs Gajah (spanish market type) and NC7 (virginia market type). However, we have encountered several problems when using such explants. The chances of obtaining a fertile plant from somatic embryos derived from immature zygotic embryos may be greater than from mature zygotic embryos, but the proportion of immature embryos that give rise to somatic embryos appears to be smaller and less predictable. The development of somatic embryos from zygotic tissue is probably optimal only at certain stages of development. Since the pods develop underground, it is difficult to estimate the exact developmental stage of pods, particularly as they grow asynchronously. Thus, a bush can be pulled from the ground and have pods of varying ages of which only a few may be suitable for regeneration. Further, the use of immature seed increases the risk of contamination in tissue culture by soil micro-organisms such as fungi. The use of immature seed also necessitates the continuous planting of seed to ensure a continual supply of explant material at the correct stage. In addition, explants from immature seeds must be used almost immediately upon removal from the ground so that storage of the seeds is not possible. All of these problems can be circumvented by the use of mature seeds as the source of explants. Mature seeds are all at the same stage of development and thus behave more predictably in culture with less wastage. Importantly, they can be stored long term at 4–15°C until needed.

As one of the major outputs of ACIAR project 9439, the University of Queensland's Botany Department in collaboration with the QDPI Queensland Agricultural Biotechnology Centre have developed a reliable method to regenerate fertile transformed peanut cultivars via microprojectile bombardment of somatic embryos derived from mature seeds of cultivars Gajah and NC7 (Livingstone and Birch 1999). This protocol results in 3–6 lines per

bombardment at least 50% of the regenerated plants being fertile. Our protocol is an important advance on the method of Ozias-Akins et al. (1996) as a practical method for the routine regeneration of fertile transgenic peanut plants of different cultivars.

Briefly, zygotic embryos are sterilised, and from these, somatic embryos are initiated and multiplied until enough are available for co-bombardment with the gene for hygromycin B resistance and the gene of choice. The somatic embryos are then placed onto selective medium containing hygromycin B. The surviving transformed cells from these somatic embryos will grow into secondary somatic embryos on selection. Once they have multiplied on embryogenesis medium, the transgenic somatic embryos are placed onto regeneration medium. When the shoots are 1 cm or taller they are placed onto rooting medium. Once they have produced roots and are approximately 10 cm tall, they can be transferred to soil.

## Timetable

### Preparation of explants:

Week 1: Sterilisation of zygotic embryos and initiation of embryogenic callus.

Weeks 2–6: Development of embryogenic callus and somatic embryos.

Weeks 7–12: Maintenance and proliferation of somatic embryos.

*Bombardment of explants* can be done at weeks 7–12. We recommend the initiation of a large number of zygotic embryo explants in tissue culture to ensure as many somatic embryos as possible are available for shooting at approximately week 7. Otherwise, embryo numbers can be further increased by culturing until week 12. However, whenever possible, try to keep to a minimum the time callus cultures spend in tissue culture so as to reduce the chance of somaclonal variation.

Day 1: Bombardment of tissue and recovery.

Day 3–8: Begin selection of transformed tissue.

Weeks 8–13 to approximately weeks 20–30:

Selection and proliferation of transformed somatic embryos.

Weeks 20–30 and onwards:

Regeneration of plantlets.

Transfer to soil and acclimatisation.

## Experimental Design

A typical experiment would include the following:

(1) Somatic embryos bombarded with the plasmid(s) carrying the selectable marker gene and the gene of interest and placed onto selective medium. This is the test treatment.

(2) Somatic embryos bombarded with the plasmid(s) carrying the selectable marker gene and the gene of interest and placed onto non-selective medium. Regenerating secondary somatic embryos indicate the medium is satisfactory. Comparison with non-bombarded tissue gives an indication of the decrease in regeneration frequency caused by the bombardment.

(3) Somatic embryos not bombarded and placed onto selective medium. No secondary somatic embryos would be expected to regenerate from this tissue, therefore if embryos do

regenerate this would give a measure of the escape rate. Equivalent numbers of somatic embryos for this treatment should be included as those used in treatment (1).

(4) Somatic embryos not bombarded and placed onto non-selective medium. See (2) for reasoning.

(5) An extra control that can be included, but is usually left out, is somatic embryos bombarded with a plasmid which is only the vector base of the plasmid used in (1) and placed onto selective medium. This ensures that the genes of interest that have been engineered into the vector do not inhibit regeneration.

## Procedure

### Sterilisation of zygotic embryos

#### 1. For mature seeds:

Remove the shell and wash seed with testa still attached in 1% chlorine for 5 minutes with shaking (RT).

Wash 3 × with sterile distilled water (RT).

#### For immature seeds:

Wash pods in sterile distilled water to remove as much dirt as possible.

Leave to dry on paper towels overnight at RT.

Remove the shell and testa and wash seeds in 70% ethanol for 5 minutes (RT).

Wash 3 × with sterile distilled water (RT).

It is important not to sterilise too many seeds at any one time as this can contribute to the spread of contamination. We received seed from both glasshouse and field grown plants. This seed was not treated with fungicide dressing because this may negatively effect regeneration. For this seed, we determined that the optimum number of seeds to sterilise in one batch was 30 seeds/200 mL of sterilant. This could vary depending on how the seeds were treated and/or stored following harvest. Thus, this may have to be determined empirically.

#### 2. Under sterile conditions, excise the embryo axes from the seeds:

Transfer a few seeds into a shallow petri dish.

Have two other clean, sterile shallow petri dishes available.

Take one seed, place it in one of the other petri dishes, using a scalpel blade and forceps split the seed into two, ensuring the embryo remains intact on one of the cotyledons (Figure 1).

Check the condition of the embryo and leaflets, discard if embryo is brown.

Turn cotyledon with embryo upside down and push down with forceps to separate the embryo from the cotyledon.

Slice off the radicle end to reduce the amount of unwanted callus, i.e. remove one third of the embryo from the root pole end (Figure 1).

Transfer embryo to a clean petri dish. Keep lid on dish to prevent embryos from drying out.

Repeat for the remaining seeds in that batch.

The removal of the leaflets as well as the radicle from the explants may result in a greater proportion of embryonic axes responding to produce somatic embryos. The leaflets alone do not respond as well as the embryonic axis to embryogenesis (ACIAR PN9439 Annual Report, 1998).

# Peanut Embryo - Mature

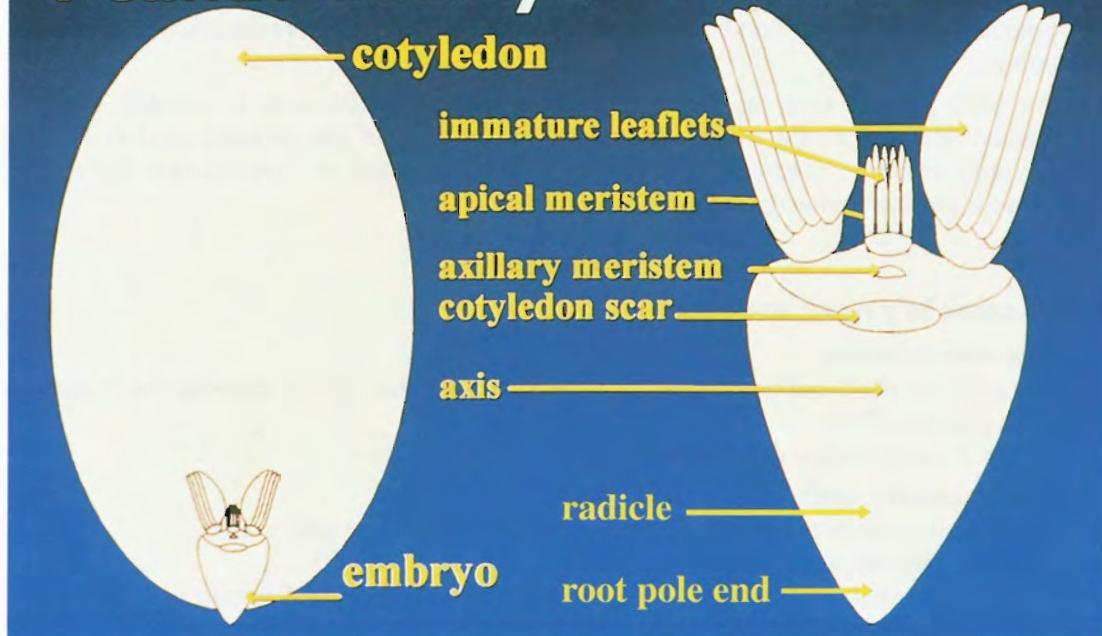


Figure 1. Schematic diagram of an embryo from a mature peanut seed. (Courtesy R.G. Birch)

3. Weigh 2 g of dichloroisocyanuric acid (DICA) into a sterile 250 mL glass bottle or equivalent (wear gloves when handling this compound).  
Dissolve in 200 mL sterile distilled water (this makes a 1% solution).  
Add a drop of Tween-20 as a wetting agent.
4. Immerse embryo axes in the fresh 1% solution of DICA in the glass bottle.  
Place on a tabletop shaker and shake at intermediate speed for 10 minutes at RT.

Do not leave for longer than 10 minutes, as this will reduce the viability of the seed.

5. Place a sterile strainer on top of a sterile beaker and strain seeds.  
Wash seeds with sterile water by pouring water over the top of the seeds held within the strainer.  
Repeat washes 2 times.
6. Place embryos into a sterile petri dish keeping lid on to prevent drying.

## Initiation and maintenance of embryogenic callus

7. Place sterilised embryos onto MS5PG medium (mature seeds) or MS1PG (immature seeds).  
Use about 10 seeds per plate.  
Seal petri dish with Micropore™ tape and label plate.  
Place at 28°C in the dark.

MS5PG is better than MS3PG as the initiation medium for mature seed explants since it will yield a higher number of embryos per explant. This medium also gives rise to less non-regenerable, mucilaginous and white friable callus than MS3PG (see notes below). Embryos have also been shown to develop from Indonesian cultivars on MS with 2% sucrose and 16 µM picloram (ACIAR PN 9349 Annual Report, 1998). Peanut somatic embryos MUST develop in complete darkness or they will go green/yellow which will make them unsuitable. Somatic embryos developed in the dark should be a milky white in colour.

The usefulness of several auxins has been compared for the development of somatic embryos in peanut. Generally, 2,4-D, NAA and picloram are all capable of allowing embryogenesis to occur. The relative effectiveness of each auxin appears to be related to the starting explant and cultivar (Dewi et al. 1997; Eapen and George 1993; Baker and Wetzstein 1998). Picloram has proven to be most suitable for embryogenesis of all cultivars tested, both spanish and virginia market types, using mature embryonic axes as explants.

Cultivars will vary as to the efficiency with which they will initiate embryos. In our hands, cvs Gajah and NC7 are quite efficient while cv. Streeton (virginia market type) was less efficient i.e. fewer explants developed embryos and fewer embryos were formed per explant. If the cultivar you are working with seems inefficient, the number of embryos for shooting can be boosted by initiating more explants to start with.

8. Subculture wanted callus (Figure 2) (cut away unwanted types — see notes below) every 2 weeks until the embryogenic callus forms. Again, seal plates with Micropore™ tape, label and place at 28°C in the dark.

Embryogenic callus can be left for many months without subculturing and still be rescued but, of course, this will greatly retard proliferation. Without attention, the cultures will eventually die. Obviously, regular subculturing is preferred. If subcultured every 2 weeks you should see a 2–3 fold increase in callus mass after each subculture.

There are several types of callus associated with embryogenic callus and it is necessary to avoid subculturing these:

1. Grey, soft callus which is not common, except on cultures neglected for long periods.
2. White, fluffy callus which is usually found only at the initiation stage on the embryo axis.
3. Brown/grey mucilaginous callus found at all stages of culture. This is the most troublesome callus and the main reason for subculturing so frequently (Figures 2, 3)

Embryogenic callus is easily distinguished from other types because it is hard. Somatic embryos are either creamy or white and some degree of differentiation is always evident including the presence of somatic embryos in various stages of development.

9. When embryogenic callus forms (4–6 weeks), a few somatic embryos will be seen. Subculture onto MS3PG (medium for proliferation and maintenance of somatic embryos) until there are sufficient somatic embryos for bombardment.
10. Maintain callus on MS3PG by subculturing every 2–3 weeks until ready for bombardment (Figure 2).



**Figure 2.** 'Good' embryogenic callus with somatic embryos.

### Bombardment of callus

Embryogenic callus is ready for bombardment at any stage after its formation but it is not worth bombarding until enough has formed to allow bombardment of at least 10 plates (each plate will contain about 10 cm<sup>2</sup> of callus).

11. 2–4 hours prior to bombardment, place callus in the middle of a petri dish containing osmotic medium MS3PGO.

Any area within the diameter of the baffle is OK but if the amount of callus available is limited then it is better to spread the tissue over more plates rather than risk all for one shot.

12. Weigh 30 mg of tungsten in a sterile 1.5 mL Eppendorf tube.

Defrost 0.1 M spermidine stock and the DNA you are going to shoot into the peanut tissue and place them on ice in preparation for the subsequent steps.

Have 2.5 M CaCl<sub>2</sub> ready at RT for the later steps also.

Any more than 30 mg is a waste of tungsten. Ensure the tungsten is stored under vacuum when not in use. If the tungsten is old and/or if you are working in a humid environment, there is a chance the



**Figure 3.** Sub-culturing embryos on a petri dish.

tungsten will clump in the later stages of preparation. If the tungsten has clumped, do not use it. Shooting large clumps into plant tissue will cause severe tissue damage such that the tissue may not survive and the chances of regenerating transformed tissue will be minimal.

13. To sterilise the tungsten, add 1 mL 70% ethanol and vortex.  
Centrifuge briefly in a microfuge to bring the tungsten to the bottom of the tube.  
Under sterile conditions, remove the alcohol with a pipette and add 1 mL of sterile distilled water.  
Centrifuge for 20–30 seconds in a microfuge and remove the water.  
Repeat this twice more.  
Finally, resuspend the tungsten to a final concentration of 100 mg/mL in sterile distilled water (i.e. 30 mg tungsten in 300  $\mu$ L water).  
Vortex the tungsten suspension well and add 25 L to a fresh, sterile 1.5 mL Eppendorf tube.
14. Ensure DNA suspension and 0.1 M spermidine are defrosted and cold.  
Quickly add 2.5  $\mu$ L of each plasmid (plasmid stock is ~ 0.7 mg/mL) followed by 25  $\mu$ L of 2.5 M  $\text{CaCl}_2$  (kept at RT).  
Vortex to mix but not at high speed or some of the mixture may adhere to the sides of the tube.

The DNA solution MUST be sterile. If unsure, re-precipitate and resuspend in sterile distilled water under sterile conditions.

The hygromycin B resistance gene and the gene of interest can be on separate plasmids or on the same plasmid. The co-bombardment frequency, if they are on separate plasmids, should be ~90%. Obviously, the co-expression frequency will be less than that.

15. Quickly add 10 µL of 0.1 M spermidine and vortex briefly to mix.
16. Allow to stand on ice for 10 minutes or until the tungsten has settled out of the suspension.
17. Remove 50 µL of the supernatant and discard.
18. Vortex briefly to resuspend tungsten/DNA mixture and load 4 µL of suspension onto the centre of a sterile 1 mm syringe filter holder for each bombardment.
19. Just before bombardment remove the lid from the petri dish containing the tissue to be shot and place a baffle over the callus.  
Place petri dish with baffle inside the gun chamber.
20. Fix syringe filter holder to Luer-lock adaptor.  
Close the chamber.  
Evacuate to ~28 mm Hg and fire (pressure of 800 kPa and pulse of 50 millisecs) to bombard tissue.  
Remove the baffle and replace with the petri dish lid. (Figure 4).

Some of the parameters that can affect the efficiency of gene delivery include the pressure and the distance of the tissue from the syringe filter holder inside the gun. We use a particle inflow gun (Finer et al. 1992) at a distance of 7–10 cm at the pressure mentioned above. This may need to be optimised for the gun set-up. This distance can be optimised by shooting a reporter gene such as the β-glucuronidase (*uidA*), luciferase (*luc*) or the green fluorescent protein (GFP) gene and monitoring the expression of the gene product (GUS, luciferase or GFP). In the case of GUS, the histochemical stain (Jefferson 1987) for GUS activity gives a blue spot wherever a *uidA* gene has been shot into a cell and expressed. In the case of GFP, microscopic detection of fluorescence indicates cells which are expressing this protein (Chalfie et al. 1994) The success of transformation depends on the balance between optimising the number of cells receiving DNA and expressing it, and minimising the number of cells that will die as a result of the shooting. Thus, it is not always the case that the highest number of hits (i.e. blue or fluorescent spots) will correspond to the greatest number of transformants regenerated. It may be that the fewer the spots, the better the regeneration frequency.

21. Leave the bombarded callus on the osmoticum medium for 2–4 hours after bombardment.

It is important to cover the petri dishes containing the bombarded tissue with aluminium foil to ensure the tissue is not exposed to too much light during this recovery period.

22. Transfer bombarded embryos to fresh MS3PG.  
Seal plates with Micropore™ tape, label and place at 28°C in the dark.

## Selection

23. After 3–7 days on MS3PG, separate small clumps of callus and place onto the selective medium, MS3PGH<sub>20</sub>.

Again, seal plates with Micropore™ tape, label and place at 28°C in the dark.

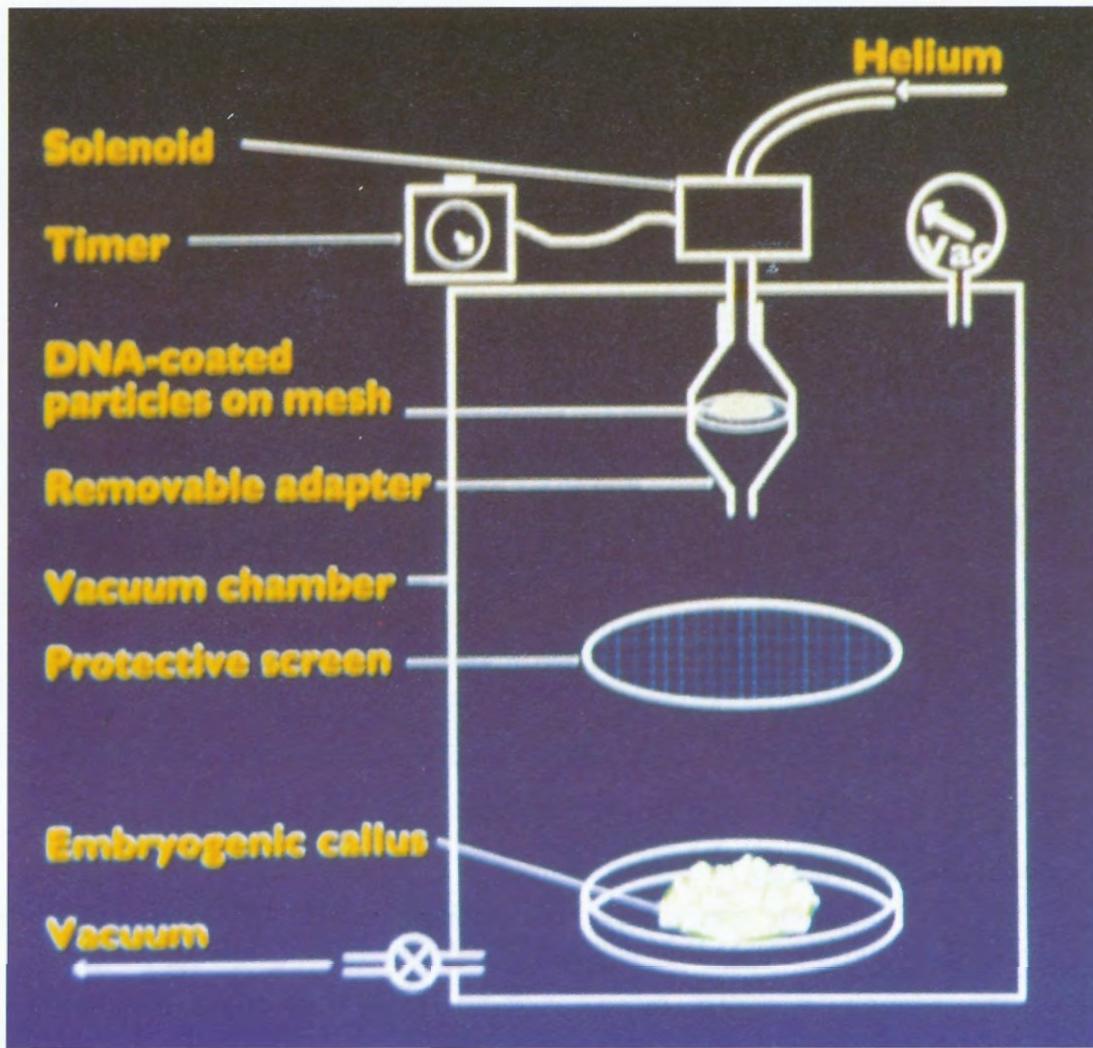
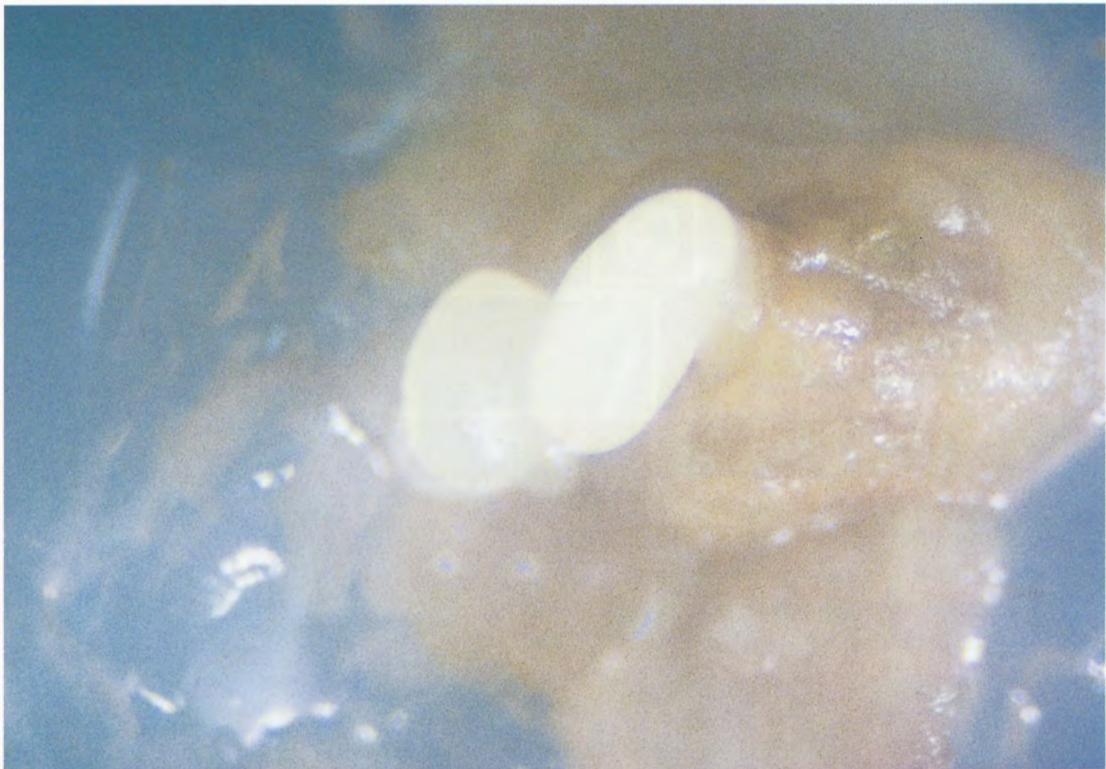


Figure 4. Diagram of a 'particle inflow gun' apparatus. (Courtesy R.G. Birch)

- Continue subculturing every 2–3 weeks until there are enough somatic embryos to begin regeneration.

Peanut tissue shows rapid necrosis, dying within 4 weeks on 20 mg/L hygromycin B. Even if all the tissue has turned brown and looks dead, it is likely that there are transformed cells within the brown tissue. Persist with your subculturing of this seemingly dead tissue as the transformed tissue will eventually emerge (Figure 5). You will recognise this tissue as it is hard and milky white in colour. In callus bombarded with pHyg<sup>r</sup> (Finer et al. 1992) small regions of emerging resistant tissue should be evident from 8–10 weeks and can be increased in volume after this time. Each somatic embryo that regenerates following bombardment will represent an individual transformation event or line. Thus, a grid marked on the bottom of the petri dish can be useful to more easily determine individual lines; 30–40 squares should be enough for each bombardment.



**Figure 5.** Embryos emerging from dead callus on antibiotic selection.

## Regeneration and rooting

25. (Optional) When ready to regenerate, place embryos onto MS3PG supplemented with 0.2 M D-sorbitol and 0.2 M D-mannitol (MS3PGO) for 4 hours, in the light.

In the initial stages of developing this protocol, we included this step. We no longer use this treatment; however, if you are having trouble getting embryos to advance to the next stage, try this. A dehydration step during regeneration of somatic embryos has been shown to increase regeneration from embryogenic callus of peanut but conventional methods of desiccation take many days and can be difficult to control. A short incubation on osmotic medium provides a convenient and reproducible means of controlled embryo desiccation to enhance subsequent regeneration. We have used it for cv. NC7 but not for other cultivars.

26. Transfer individual embryos to MSC medium and culture under a 16 hour photo period at 28°C for four weeks.
27. Transfer embryos to MS10 medium and continue to grow under a 16 hour photo period at 28°C, subculturing every 2 weeks.

Embryogenic callus develops asynchronously and produces somatic embryos, which vary in morphology (Figure 6). Some embryos may regenerate on MSC, others on MS10 (Figure 7). The callus becomes organogenic on MS10 and the quality of shoots regenerated is higher if they regenerate on

this medium. A gradual decline in regeneration efficiency does occur over time in culture, so the duration of callus proliferation should be minimised to ensure the callus can convert into shoot structures with greatest efficiency and quality. There is no difference in regeneration frequency between torpedo or fused embryos for cvs NC7 and Gajah, although others have found peanut embryo morphology to influence regeneration efficiency (Baker and Wetzstein 1992; Baker et al. 1995).

28. Once shoots have developed (1–5 cm) they can be excised by cutting away any root-like structures or callus and placing them on MSOR or MSO medium.  
Continue growth under a 16 hour photo period at 28°C.

Shoots should produce roots within 1 month and sometimes elongate partially on both media (Figure 8). For most lines it will be necessary to place shoots directly on MSO i.e. no hormones.

29. Once roots have formed, transfer plantlets to larger containers of MSO medium to allow shoots to develop and the plants to elongate (Figure 9).  
Grow under a 16 hour photo period at 28°C.  
Plants can be multiplied through nodal cuttings.  
A plant suitable for transfer to soil is shown in Figure 10.



Figure 6. Different shapes of embryos observed.

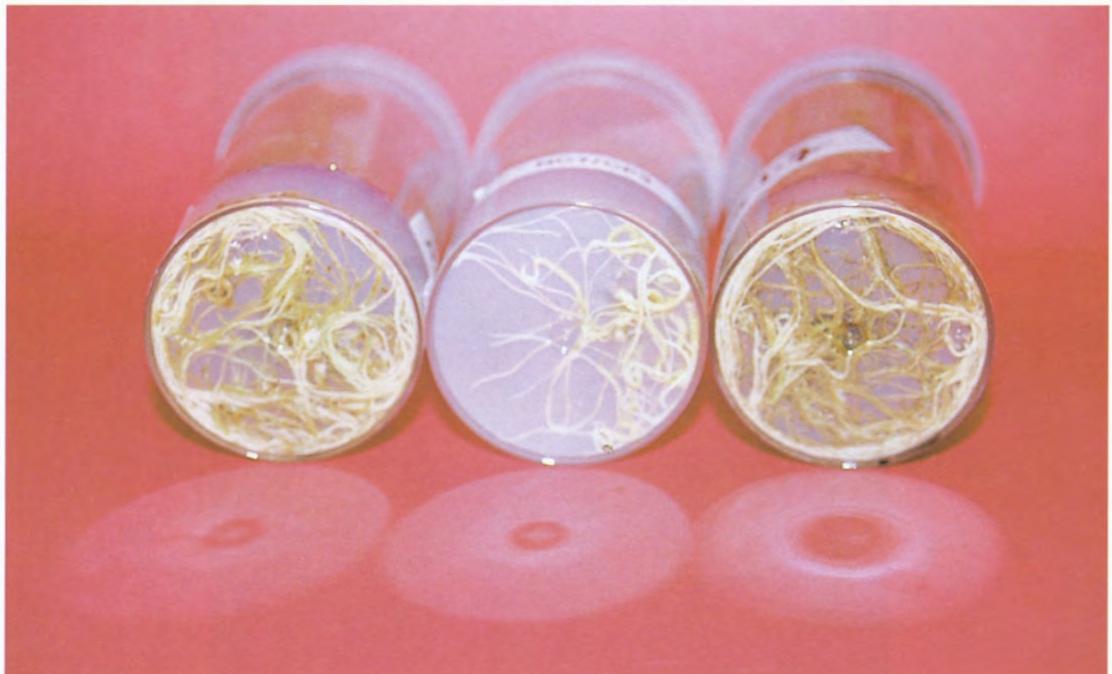


**Figure 7.** Shoots emerging from transformed callus on a tissue culture plate.

You may notice the proliferation of callus at the crown (root-shoot junction) of the plantlet. It is likely that the roots growing from this area are not attached directly to the shoot. In this case, the plantlet will not grow once it is in soil since the flow of nutrients is likely to be interrupted by the callus. The formation of this callus may be likely to occur on certain cultivars; we noticed it more commonly on cv. Gajah than on cv. NC7 for example. To eliminate this callus, excise the shoot and try rooting again on hormone-free medium.

## Transfer to soil and acclimatisation

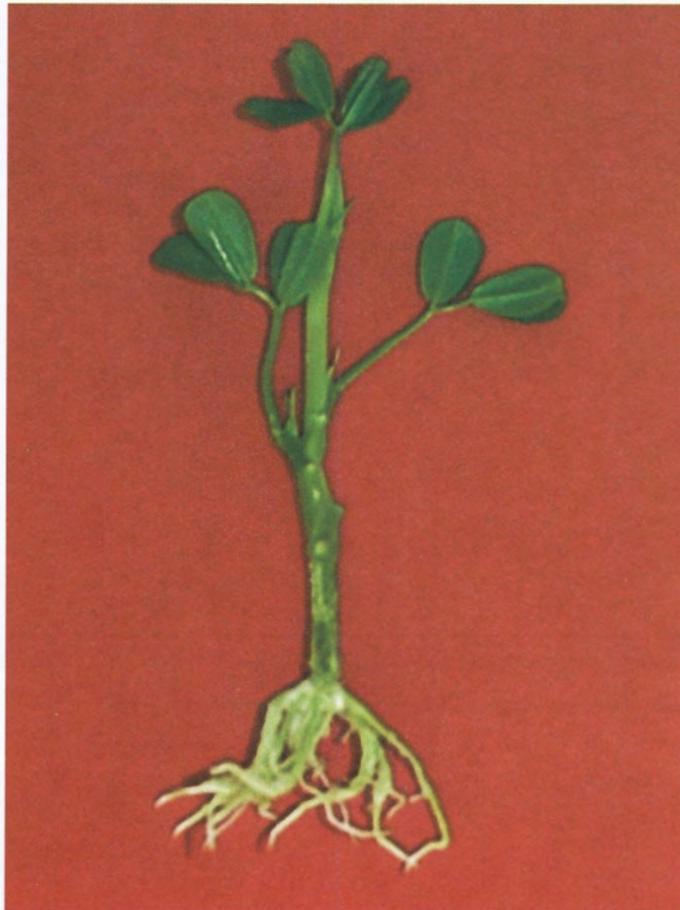
Tissue culture regenerated plantlets do not photosynthesise nor do they have a waxy cuticle to protect them from the environment. Thus, they are vulnerable to dessication and pathogen attack when transferred to soil. The purpose of acclimatisation of tissue culture plants is to gradually condition them to lower humidity levels, natural sunlight and to obtaining nutrients from soil and via photosynthesis. Among other factors, successful acclimatisation depends on the season when plants are transferred into the glasshouse. In Australia, acclimatisation during the cooler times of the year was often unsuccessful and worked much better during summer. A humidifying/misting chamber would be the most effective and convenient way of acclimatising peanut plants and this would circumvent the problems we encountered when using plastic bags as individual covers (Figure 11). Four main issues impact on the process of acclimatisation:



**Figure 8.** Root development on transgenic peanut plantlets in tissue culture.



**Figure 9.** Growth of peanut shoots into plantlets in tissue culture.



**Figure 10.** Peanut plantlet suitable for transfer to soil.

1. **Plantlet quality.** Peanut plantlets should be 8–10 cm tall with at least 3 sets of dark green expanded leaves, a vigorously growing shoot tip and at least one root, which is attached directly to the stem. Smaller peanut plantlets have a low survival rate and are more susceptible to fungal contamination.
2. **Soil type.** Soil should be well drained and not over-watered. We use a steam-sterilised mix of UC-B: sand (1:1).
3. **Temperature and humidity.** Stable temperatures of 25°C–28°C are optimal for acclimatising peanut plants. Initially, plants require high humidity conditions to mimic the tissue culture environment. Plants will adjust to the gradual lowering of humidity as their roots adjust to the soil.

To decrease the chance of microbial contamination of cultures all work with soil should be done well away from the tissue culture facilities and preferably not directly before tissue culture work.

**30. Remove plant in agar plug from tissue culture vessel.**

Remove agar gently from roots with hands under running tap water being careful not to damage roots. Remaining agar is a good nutritional source for soil micro-organisms and needs to be thoroughly washed off.



**Figure 11.** Slow reduction in humidity levels using pots covered with plastic sleeves during acclimatisation of peanut plantlets in soil.



**Figure 12.** Transgenic peanut plants growing in soil-containing pots in a glasshouse.

31. Transfer plantlet to soil mix in a 100 mL pot and cover with a plastic bag (Figure 11). Ensure all soil is removed from leaves and that leaves do not touch the bag. Condensation causes the leaves to become water-soaked and highly susceptible to fungal infection.
32. Over the next 1–2 weeks, place small holes in the bags and keep adding more holes or enlarging existing ones to gradually reduce humidity levels. The extent of condensation on the inside of the bags is a good indicator of the humidity level. Once the inside of the bag has been dry for a week, the bags can normally be removed and plants should be acclimatised. Once the plants have been established (Figure 12), they can be transplanted to larger pots for seed production.

Placing holes in the mid-section of the bag rather than the top near the shoot tips appears to improve survival rates. This prevents the shoot tips from wilting which often results in the death of the plant.

## Reagents and Equipment

### **Sterilisation of zygotic embryos**

Absolute alcohol for sterilising tools (i.e. if not using Bacti-Cinerator).

Bunsen burner or Bacti-Cinerator in laminar flow hood.

Dichloroisocyanuric acid powder (DICA).

For immature seeds: paper towels, 70% ethanol.

For mature seeds: 1% chlorine.

Laminar flow hood.

Marking pen.

Mature or immature peanut seeds.

Micropore™ tape.

Non-sterile tissue culture jar.

Petri dishes.

Scalpel blade and other tissue culture tools such as forceps.

Sterile 250 mL glass bottle or equivalent.

Sterile blotting paper.

Sterile distilled water.

Sterile petri dishes.

Sterile strainers.

Sterile tissue culture jar.

Table top shaker.

Tween-20.

### **Initiation and maintenance of embryogenic callus**

Sterilised embryos.

MS5PG medium (mature seeds) or MS1PG (immature seeds) plates.

Laminar flow hood.

Marking pen.

Plant growth chamber at 28°C either dark or with dark box.

Micropore™ tape.

MS3PG plates.

### **Bombardment of callus**

0.1 M spermidine.

2.5 M CaCl<sub>2</sub>.

70% ethanol.

An operational microprojectile gun with appropriate accessories such as syringe filter holders, baffles, Luer-lock adaptors, helium tank, pressure gauge etc; we use a particle inflow gun (i.e. a helium driven gun) but any gun design would be suitable.

Automatic pipettes for measuring 10, 20, 100 and 1000 µL

Embryogenic callus.

Ice and ice bucket.	<b>Regeneration and rooting</b>
Marking pen.	70% ethanol for use in laminar flow hood.
Microfuge.	Absolute alcohol for sterilising tools (i.e. if not using Bacti-Cinerator).
Micropore™ tape.	Bunsen burner or Bacti-Cinerator in laminar flow hood.
MS3PG plates.	Laminar flow hood.
MS3PGO plates.	Marking pen.
Plant growth chamber at 28°C either dark or with dark box or tin foil for wrapping plates.	Materials for assaying transient gene expression eg GUS histochemical stain — optional.
Sterile 1.5 mL Eppendorf tubes.	Micropore™ tape.
Sterile distilled water.	MS10 plates.
Sterile plasmid DNA ~0.7 µg/µL.	MS3PGO plates — optional.
Tungsten (1 µm particles).	MSC plates.
Vortex shaker.	MSO medium in tissue culture vessels.
<b>Selection</b>	MSOR medium in tissue culture vessels.
70% ethanol for use in laminar flow hood.	Plant growth chamber at 28°C with 16 hr photo period.
Absolute alcohol for sterilising tools (i.e. if not using Bacti-Cinerator).	Sterile scalpel blades with handles, sterile forceps etc.
Bunsen burner or Bacti-Cinerator in laminar flow hood.	Transformed somatic embryos/shoots.
Laminar flow hood.	<b>Transfer to soil and acclimatisation</b>
Marking pen.	100 mL pots and larger.
Materials for assaying transient gene expression eg GUS histochemical stain — optional	Glasshouse suitable for transgenic plants.
Micropore™ tape.	High quality transformed plantlets, rooted in tissue culture vessels.
MS3PGH <sub>20</sub> . Plates.	Liquid fertiliser.
Plant growth chamber at 28°C either dark or with dark box.	Plastic bags or tissue culture vessels for covering plantlets.
Stereomicroscope capable of ~5–50 × magnification with black platform and adequate light source for observing cultures; the use of such a microscope is not essential for the regeneration of peanut but observing the formation of embryos will greatly enhance the operator's culturing skills.	Plastic container such as a bucket.
Sterile scalpel blades with handles, sterile forceps etc.	Soil, autoclaved mix of 1:1, UC-B mix:sand.
	Tap water.

## **2.2 *Agrobacterium tumefaciens*-mediated transformation of peanut**

Several reports have been published describing *Agrobacterium*-mediated transformation of peanut using a variety of explants with varying success at regenerating fertile transgenic plants.

Peanut was shown to be a suitable host for *Agrobacterium tumefaciens* by Lacorte et al. (1991). Using a variety of wild type strains, they were able to induce crown gall formation in a range of Brazilian cultivars including cv. Tatu (Valencia botanical type), cv. Tupa (Valencia), cv. Tatu Branco (Spanish), cv. Tatui (Spanish) and cv. Penapolis (Virginia) with the best wild type strain being A281. This strain appeared to induce the largest tumours with the highest frequency. The first report of peanut transformation using a disarmed *Agrobacterium* strain was by Eapen and George (1994), who described the use of strain LBA4404 containing the plasmid pBI121 to inoculate leaves of subspecies fastigata variety TAG-24. Thus, transgenic tissue was selected using kanamycin and the success of gene delivery was determined by GUS histochemical assay. However, they did not report the production of fertile plants. The most successful method published to date appears to be that of Cheng et al. (1996; 1997) using leaf sections from cv. New Mexico Valencia A with the strain EHA105 containing plasmid pBI121. While they were able to regenerate fertile plants, this is a technically difficult procedure that relies heavily on operator skills and optimal laboratory conditions.

The success of *Agrobacterium*-mediated transformation appears to be heavily influenced by the peanut cultivar, the strain of *A. tumefaciens* used, the plasmid that the bacterium carries, as well as the co-cultivation conditions and the efficiency of the regeneration system. There have been preliminary studies reporting the optimisation of co-cultivation conditions (Cheng et al. 1994; Newton 1997; Egnin et al. 1998), comparing *Agrobacterium* strains and peanut cultivars (ACIAR PN9439 Annual Report 1997; Egnin et al. 1998) and describing the potential of various regeneration systems for adaptation to *Agrobacterium*-mediated transformation (De Freitas et al. 1997; Sarker et al. 1997). The majority of publications report the use of the cultivar New Mexico Valencia A (Cheng et al. 1996, 1997; Egnin et al. 1998). Indeed, this cultivar was found to be more amenable to DNA delivery than runner varieties such as cv. Florunner (Egnin et al. 1998). There have been few reports concerning commercially important varieties (Newton 1997; Fang et al. 1999). Most studies have used the bacterial strains EHA101 or EHA105 (Hood et al. 1986), derivatives of A281 (Franklin et al. 1993; McKently et al. 1995; Cheng et al. 1996; Egnin et al. 1998). Although, DNA delivery has also been achieved using other strains such as LBA4404 (Franklin et al. 1993; Eapen and George 1994) and AGL1 (Newton 1997). The most commonly used plasmid has been pBI121 (Eapen and George 1994; Cheng et al. 1996; Sarker et al. 1997) which allows selection of transgenic tissue on kanamycin-containing media and its analysis by GUS histochemical assay. Other plasmids have also been used such as pROA93 (with CaMV 35S-*nptII* and CaMV 35S-*uidA* genes) (Fang et al. 1999) and pTOK233 (Newton 1997; ACIAR PN9439 Annual Report 1998). Use of pTOK233 has allowed *Agrobacterium*-mediated transformation of the recalcitrant monocot rice (Hiei et al. 1994). We have shown that this plasmid gave the highest transient gene expression and is likely to be the best plasmid to use for peanut transformation (Newton 1997). Most reports describing *Agrobacterium*-mediated transformation of peanut have used kanamycin as the selection agent (Franklin et al. 1993;

Eapen and George 1994; Cheng et al. 1996, 1997; De Freitas et al. 1997). However, we have found hygromycin B to be a more suitable selective agent for cvs Gajah and NC7 (Newton 1997; ACIAR PN9439 Annual Report 1998).

The production of transgenic peanuts avoiding tissue culture altogether has also been described (McKently et al. 1995). In this study, mature embryo axes were inoculated and allowed to germinate to produce seed. Analysis of the T1 seedlings showed 9% to be transformed. However, it is quite possible that many of these plants were chimeras since there was no description of inheritance into the T2 generation.

We present protocols for *Agrobacterium*-mediated peanut transformation using both leaflets and somatic embryos as explants. The regeneration of plantlets from leaflets occurs via organogenesis, while the regeneration from somatic embryos occurs via embryogenesis followed by organogenesis. In our laboratory, these transformation protocols have not, as yet, proven themselves to be as efficient as microprojectile bombardment of embryogenic callus but may do so with further experimentation. We offer these alternative protocols since they may be more suitable for laboratory conditions in developing countries. For example, you may not have access to a microprojectile gun, but can grow agrobacterial cultures. If you are interested in using these alternative approaches, it may be necessary for you to do some initial experiments to improve their efficiency in your laboratory environment. The peanut cultivar, agrobacterial strain and/or plasmid are only a few parameters that may need optimising.

### **(A) Transformation using leaflets as explants and regeneration via organogenesis**

This protocol is an adaptation of the regeneration method described by Livingstone and Birch (1995). We have found the efficiency of this method to be  $3.8 \pm 1.8\%$  for stable transformants (Newton 1997).

#### **Timetable**

- Day 1: Sterilisation and sowing of peanut seed to prepare explants.  
Starting *Agrobacterium* culture.
- Day 2: Setting up *Agrobacterium* culture for inoculation of explants.
- Day 3: Preparation of *Agrobacterium* culture for inoculation.  
Preparation of explants.
- Day 5: Recovery of explants from co-cultivation.
- Day 9–10 and Onwards:  
Selection, regeneration and rooting of transformed plantlets.  
Transfer to soil and acclimatisation of transformed plants.

#### **Experimental Design**

A typical experiment would include the following:

Leaflets from 2 day-old germinated peanut seeds are used as explants with 1 seed providing 6 leaflets.

- (1) Explants infected with *Agrobacterium* carrying the binary plasmid of interest and placed onto selective medium. This is the test treatment.
- (2) Explants infected with *Agrobacterium* carrying the plasmid of interest and placed onto non-selective medium. Regenerating shoots indicate the medium is OK. Comparison with tissue not infected with *Agrobacterium* gives an indication of the decrease in regeneration frequency caused by infection with *Agrobacterium*.
- (3) Explants not infected with *Agrobacterium* and placed onto selective medium. No shoots would be expected to regenerate from this tissue. Therefore, if shoots DO regenerate, this would give a measure of the escape rate. For this treatment, equivalent numbers of seeds should be included to those used in treatment 1.
- (4) Explants not infected with *Agrobacterium* and placed onto non-selective medium. See (2) for reasoning.
- (5) An extra control that can be included, but is usually left out is explants infected with *Agrobacterium* carrying a plasmid, which is only the vector base of the plasmid used in (1). If you suspect that the genes you are trying to transfer are inhibiting regeneration, you may want to include this control. (This control is not necessary during initial transformation experiments).

## Procedure

### Establishing an *A. tumefaciens* culture

1. Streak out a 16-streak plate of *A. tumefaciens* carrying the plasmid of choice onto a YEB agar plate containing the appropriate antibiotics. Grow for 2–3 days at 28°C to obtain single colonies.

When *Agrobacterium*-mediated transformation experiments are ongoing in the laboratory, it is important to have healthy, fresh cultures available. This can be achieved by streaking out plates of *Agrobacterium* weekly and storing at 4°C for up to 2 weeks.

Specific antibiotics are used to select for the *Agrobacterium* carrying the plasmid of interest. For example, the plasmid pBI121 is based on the vector pBIN19 and as such has a bacterial gene conferring resistance to kanamycin. Contamination of *Agrobacterium* cultures by *Escherichia coli* carrying similar plasmids can be avoided by including 20 mg/L rifampicin, although the use of this antibiotic is optional. Thus, for pBIN19-based plasmids such as pBI121 you would use 50 mg/L kanamycin and 20 mg/L rifampicin to grow up this culture.

A variety of *A. tumefaciens* strains and plasmids have been used to deliver genes to peanut tissue. The wild type strain A281 was shown to be best at causing gall formation (Lacorte et al 1991), thus we recommend using disarmed strains derived from this such as EHA101, EHA 105 (Hood et al. 1986) or AGL1. While it appears that most plasmids are capable of delivering genes, which are expressed transiently, the conversion of transient expression to stable transgene expression appears very low for the majority of plasmids used to date. In our hands, we have found that the superbinary plasmid pTOK233 (nos-nptII; CaMV 35S-uidA; CaMV 35S-hpt) (Hiei et al. 1994) is more effective at delivering DNA to peanut cells than any other plasmid we have tried. It carries an extra copy of the virG gene, which is a transcriptional enhancer of the *Agrobacterium* virulence gene system. Use of this or an equivalent plasmid may be necessary for the development of a routine, efficient method.

## **Day 1: Sterilisation and sowing of peanut seed**

About 6 explants can be derived from 1 seed, thus for 360 explants 60 seed are sterilised.

1. Wash seeds in 1% (w/v) active chlorine containing 1 drop/L of Tween-20 for 5 minutes with shaking (RT). During this period, the sterilising solution is filtered off and replaced 3 times with fresh sterilant. Do this by pouring seeds/sterilant into a sterile strainer such as a tea strainer placed over a sterile beaker, return seeds to sterilising container and replace sterilant. Work quickly. Do not leave for longer than 5 minutes as this will reduce the viability of the seed.

Wash seeds 3 × with sterile distilled water (RT). Do this by pouring off the remaining sterilant through the strainer, return seeds to container and replace sterilant with sterile distilled water. Shake well to thoroughly wash seeds. Repeat twice, leaving a little water in the jar after the final wash to prevent dehydration of the seeds (~600–800 mL in total). Check the condition of the embryo and leaflets.

Discard any half seeds or seeds where the embryo and leaflets look damaged or brown.

As described for microprojectile bombardment of embryogenic callus, we determined that the optimum number of seeds to sterilise in one batch was 30 seeds/200 mL of sterilant. This could vary depending on how the seeds were treated and/or stored following harvesting. Thus, this proportion may have to be determined empirically under your laboratory conditions.

2. Transfer about 10 seeds into a sterile petri dish.

Have 2 other clean, sterile petri dishes available.

Take 1 seed, place in one of the other petri dishes; using a scalpel blade and forceps split the seed into two, ensuring the embryo remains intact on one of the cotyledons.

Place the cotyledon with the attached embryo into the remaining petri dish.

Repeat for all the seeds until finished.

3. Weigh 2 g of dichloroisocyanuric acid (DICA) into a sterile 250 mL glass bottle or equivalent (wear gloves when handling this compound).

Dissolve in 200 mL sterile distilled water (this makes a 1% solution).

Add a drop of Tween-20 as a wetting agent.

4. Place split seed halves with embryo prepared in 2. in a sterile jar and add the DICA/Tween solution.

Place on table top shaker and shake at intermediate speed for 10 minutes at RT. Do not leave for longer than 10 minutes as this will severely reduce the rate of germination by damaging the embryos.

5. Place a sterile strainer on top of sterile beaker and strain seeds.

Wash seeds with sterile water by pouring water over the top of the seeds held within the strainer.

Repeat washes 4–5 × (~600–800 mL in total).

Place embryos into a sterile petri dish keeping lid on to prevent drying out.

6. Place 1–2 sheets of sterile blotting paper into sterile petri dish. Place strainer, holding seeds, onto blotting paper to remove excess liquid.

7. Place embryos onto MSO medium laying cotyledon horizontal with the embryo just above the medium. Have about 10 seeds per plate.  
Label plate and seal with Micropore™ tape.  
Leave in growth chamber at 28°C in the light under a 16 hours photo period for 2 days before excising leaflets (see Day 3).

### **Day 1: Starting *Agrobacterium* culture**

8. Pour 10 mL of sterile YEB containing appropriate antibiotic(s) into a 30 mL sterile screw cap tube.  
Using a sterile wire loop, touch ~5 agrobacterial colonies on the plate and transfer to the YEB medium.  
Incubate overnight at 28°C in a shaking incubator at ~200 r/min.

A starter culture as described here is recommended to ensure the *Agrobacterium* is actively growing.

### **Day 2: Setting up *Agrobacterium* culture for inoculation of explants**

9. Measure the OD<sub>600</sub> of the starter culture.  
Have 100 mL of sterile YEB containing the appropriate antibiotics ready.  
If the culture has an OD<sub>600</sub> of 2.2, use 100 µL to inoculate the 100 mL of YEB. If the OD<sub>600</sub> is more or less, adjust the volume accordingly.  
Grow this culture overnight (15–20 hr) at 28°C in a shaking incubator at ~200 r/min.

To ensure you have a culture at the right stage of growth, you may wish to set up more than one starter culture and culture for inoculation, since each culture will probably be at a slightly different concentration after the growth period. It may be useful to analyse the growth pattern of the *Agrobacterium* under your laboratory conditions by establishing a growth curve prior to the transformation experiment. This will give you an idea of the time it takes a certain amount of inoculum in a particular volume to reach the desired density. The growth curve is plotted as time against OD<sub>600</sub>.

### **Day 3: Preparing the *Agrobacterium* culture for inoculation**

10. Measure the OD<sub>600</sub> of the overnight culture. It should be at early to mid log phase, about OD<sub>600</sub> = 0.3–0.5, for transformation.  
Have ready cold MS3a with/without acetosyringone (AS).

The optimum condition for *Agrobacterium* is to be in mid-log phase of growth. If it has reached stationary phase, its ability to transform peanut cells is reduced. Therefore, it is better if the OD reading is at the lower end of the range.

11. Cool cultures to 4°C for 20 minutes by either placing in a refrigerator or on ice.

Chilling helps the cells to spin down more efficiently, although this step is optional.

12. Pour the cultures into sterile centrifuge tubes suitable for a Sorvall SS34, Beckman JA20 or a similar rotor.  
Pellet the cells by centrifuging at 4500 r/min at 4°C for 10–15 minutes.

Pour off the supernatant from the pellet and drain upside down on blotting paper for 1 minute, ensuring the pellet does not slide from the tube.

Resuspend each bacterial pellet in 10 mL of cold MS3a medium (with or without AS), ensuring the pellet is fully resuspended either by gentle vortexing or pipetting up and down.

Re-centrifuge as before and drain off supernatant as before.

Resuspend all pellets from one 100 mL culture in a total of 10 mL of cold MS3a containing 100 µg/L AS (MS3a/AS).

The medium used to grow the *Agrobacterium* must be changed from bacterial growth medium to plant growth medium. Bacterial growth medium and any antibiotics used can inhibit plant regeneration. This switch is carried out by pelleting the bacteria, pouring off the medium and replacing it with plant growth medium.

13. Measure OD<sub>600</sub> using MS3a/AS as the blank. Dilute bacterial suspension with cold MS3a/AS to give an OD<sub>600</sub> of 1.0.
14. Leave bacterial suspension on ice until required.

### **Day 3: Preparation and inoculation of explants**

At this point, the explants to be used for inoculation with *Agrobacterium* must be prepared. These are the leaflets from the sterile peanut seed that have been germinating since day 1. Under sterile conditions, the leaflets are cut from the cotyledon and soaked in the suspension of *Agrobacterium* before being placed onto co-cultivation medium. The quality of the explants is important so be careful to choose only healthy looking seed/leaflets. Brown seeds are not suitable, nor are any that are contaminated with bacteria or fungi.

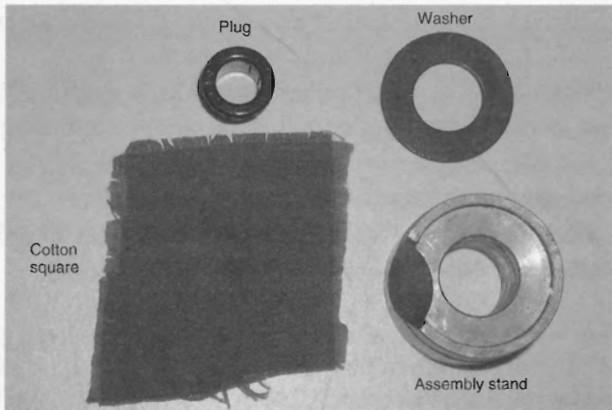
Our first attempts to develop an *Agrobacterium*-mediated transformation system for peanuts were based on the use of leaflets of 4 day-old germinated seedlings (Fang et al. 1999). Subsequently, we found leaflets from 2 day-old germinated seedlings to be superior, since these gave rise to more shoot primordia per explant (Newton 1997).

The leaflet explants are very small and may be difficult to handle. You may wish to make an apparatus in which to hold the leaflets for exposure to *Agrobacterium*. We have designed an apparatus called a 'peanut explant containment apparatus' or PECA (Figure 13). This apparatus and its components can be autoclaved and used throughout the process of inoculating explants with *Agrobacterium*.

To date, we have not distinguished the leaflet condition following germination as more or less suitable for regeneration. We have taken leaflets regardless of whether or not the seed has started to germinate but rather have ensured seeds were not brown or contaminated. Generally, all explants inoculated with *Agrobacterium* are treated together and all those not inoculated are treated together. The explants are separated after co-cultivation depending on the different selection regimes they will follow.

15. Working in a laminar flow hood, open a sterile petri dish and place lid on bench of hood with the inside up.  
Pipette 1 mL of liquid MS3a medium into the lid of the petri dish. If using a PECA, place the cotton in the petri dish base and wet with 1 mL MS3a.

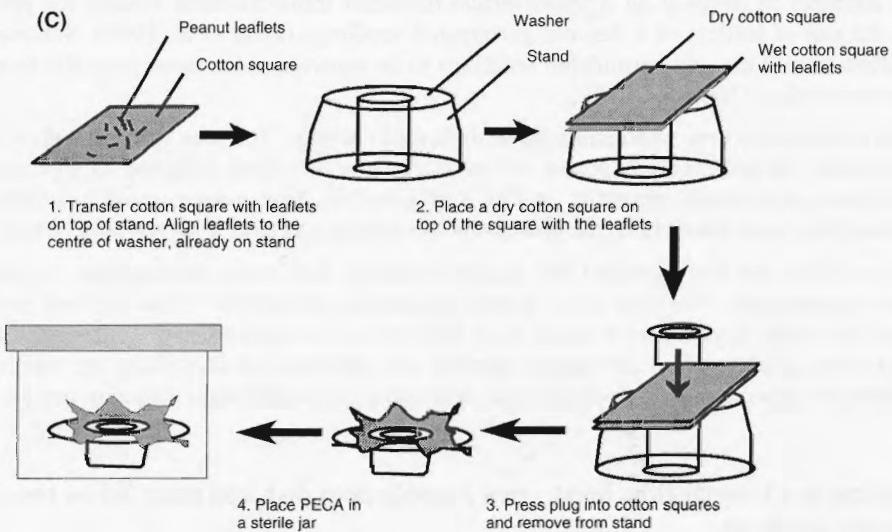
(A)



(B)



(C)



**Figure 13.** The 'peanut explant containment apparatus' (PECA) as used in *Agrobacterium*-mediated transformation of leaflets. (A) disassembled components; (B) PECA assembly; and (C) flow diagram of PECA use. (Courtesy T. Newton)

16. To the base of the petri dish, transfer a 2 day-old germinating peanut cotyledon with leaflets attached. Using a sharp, sterile scalpel blade, carefully cut the leaflets away from the cotyledon, ensuring that the leaflets are the only part removed without any of the epicotyl.

Transfer the excised leaflets to the wet cotton or MS3a medium in the petri dish base to ensure the tissue does not dry out.

Ensure that the leaflets are sliced cleanly and separated from each other.

Repeat for all the seeds, ensuring that only good quality seed and leaflets are used.

17. Without PECA: Once all the leaflets have been excised and transferred into the petri dish, pour the *Agrobacterium* suspension in MS3a into the petri dish.

With PECA: Cover leaflets on cotton piece with another piece of sterile cotton. Place cotton/leaflets into PECA and assemble. Place into a sterile screw-capped container (~30 mL) and pour in the *Agrobacterium* solution.

Seal with parafilm to ensure none of the culture is spilt.

18. Place on a tabletop shaker and agitate gently for 20 minutes at RT.

19. Without PECA: Remove the *Agrobacterium* suspension by straining leaflets with a sterile strainer.

With PECA: Pour off the majority of the *Agrobacterium* suspension and remove PECA from container.

Place sterile blotting paper into a sterile petri dish and transfer strainer with leaflets/PECA with leaflets onto this blotting paper to quickly blot off excess liquid.

If using PECA, disassemble, retaining leaflets on one of the cotton pieces.

20. Transfer infected leaflets to agar plates of MS3a/AS. If using PECA, place cotton piece leaflets-side down onto the agar and remove cotton ensuring leaflets remain on agar.

The orientation of the leaflets is not important at this stage. Also, the leaflets can be fairly crowded on the plate at this stage, but spread out as much as possible.

21. Label and seal the plate with Micropore™ tape and place into plant growth chamber for 2 days co-cultivation at 24°C under a 16 hour photo period.

If you have transformed with a reporter gene such as the *uidA* gene or GFP gene, expression of the reporter gene can be monitored after one day on MS3a and onwards.

The co-cultivation temperature of 24°C has been shown to significantly improve the efficiency of transient gene expression and hence gene delivery. Inclusion of acetosyringone in the co-cultivation medium was shown to either have no effect (Mansur et al. 1993) or double transient GUS expression (Newton 1997). This effect appears to be dependent on the peanut cultivar (cv's Gajah and NC7 were more receptive than cv. Florunner) as well as on the *Agrobacterium* strain × plasmid since LBA4404 × pTOK233 responded better than AGL1 × pKIWI105 (Newton 1997). Further, AGL1 × pKIWI105 responded better, when acetosyringone was included in the agrobacterial growth medium for 15 hours prior to co-cultivation, while LBA4404 × pTOK233 responded best when it was included in the co-cultivation medium only. Our recommendation is to include it in the co-cultivation medium, since it may improve gene delivery, and it certainly will not inhibit it. You may also wish to include it in the bacterial growth medium to ensure its effect.

## **Day 5: Recovery of explants from co-cultivation**

22. Following co-cultivation, transfer explants to MS3aCef<sub>300</sub> plates to recover and to kill off the *Agrobacterium*. Explants are placed cut edge into the medium (but not too deep!) using about 30 explants per plate.

Plates are labelled and sealed with Micropore™ tape before being placed into a plant growth chamber at 28°C under a 16 hour photo period for 45 days to recover.

At this stage, the *Agrobacterium* growth must be controlled otherwise it can overgrow and kill the plant tissue. Typically, cefotaxime (cef) is included in the medium at 300 mg/L or timentin at 150 mg/L. If both are available, they can be used together at these concentrations, otherwise use them alone. No antibiotic is added at this stage (i.e.: hygromycin or kanamycin) for the selection of transformed plant cells. This step is for the explants to recover from the co-cultivation.

## **Day 9–10 and Onwards: Selection, regeneration and rooting**

After the explants have had a chance to recover from the co-cultivation, it is time to begin the selection of transformed plant cells and to regenerate plants from them. At this point, the selective agent, i.e. hygromycin B (H), is added to the medium as well as cefotaxime and/or timentin, which is still needed to ensure the *Agrobacterium* does not grow back and kill the plant tissue.

23. Explants are transferred to MS3aCef<sub>300</sub>H<sub>20</sub> plates with the cut edge placed into the medium (but not too deep!). Place a maximum of 15 explants per plate. Plates are labelled and sealed with Micropore™ tape before being placed into the plant growth chamber at 28°C, 16 hour photo period.

We have tested kanamycin (100 and 125 mg/L), G418 (25, 50, 100 and 200 mg/L) and hygromycin B (5, 10 and 20 mg/L) as agents for the selection of transformed cells. Kanamycin was found to be ineffective as a selection agent. Both G418 and hygromycin B were found to be effective at killing untransformed cells; however, only in the presence of hygromycin B did transformed explants go on to form shoot primordia. Thus, hygromycin B is the appropriate selection agent for the regeneration of transformed peanut via organogenesis.

24. Subculture the explants onto fresh MS3aCef<sub>300</sub>H<sub>20</sub> medium every 2 weeks, and monitor the growth of shoot primordia on those explants infected with *Agrobacterium* compared with those not infected.

Primordia should appear at the cut edge of the explants that have been infected with *Agrobacterium* and placed onto hygromycin as well as those that are not on hygromycin. These may only be visible under the microscope. (Figure 14)

25. Once the explant is completely covered in shoot primordia, transfer it to MSB5Cef<sub>300</sub>H<sub>20</sub> medium and monitor the elongation and maturation of shoots.  
Subculture every 2 weeks onto fresh MSB5Cef<sub>300</sub>H<sub>20</sub> medium.

If the explants are kept for too long on MS3aCef<sub>300</sub>H<sub>20</sub>, the shoot primordia may not elongate. The shoot primordia may develop better at this stage if taken off medium containing hygromycin B. If you do remove the hygromycin B at this stage, do so only if you are confident that there has been a long

enough period of selection. This can be gauged by observing the untransformed tissue on hygromycin B containing medium. If they have all died, you can be reasonably confident that the tissues you are observing on selection are indeed transformed.

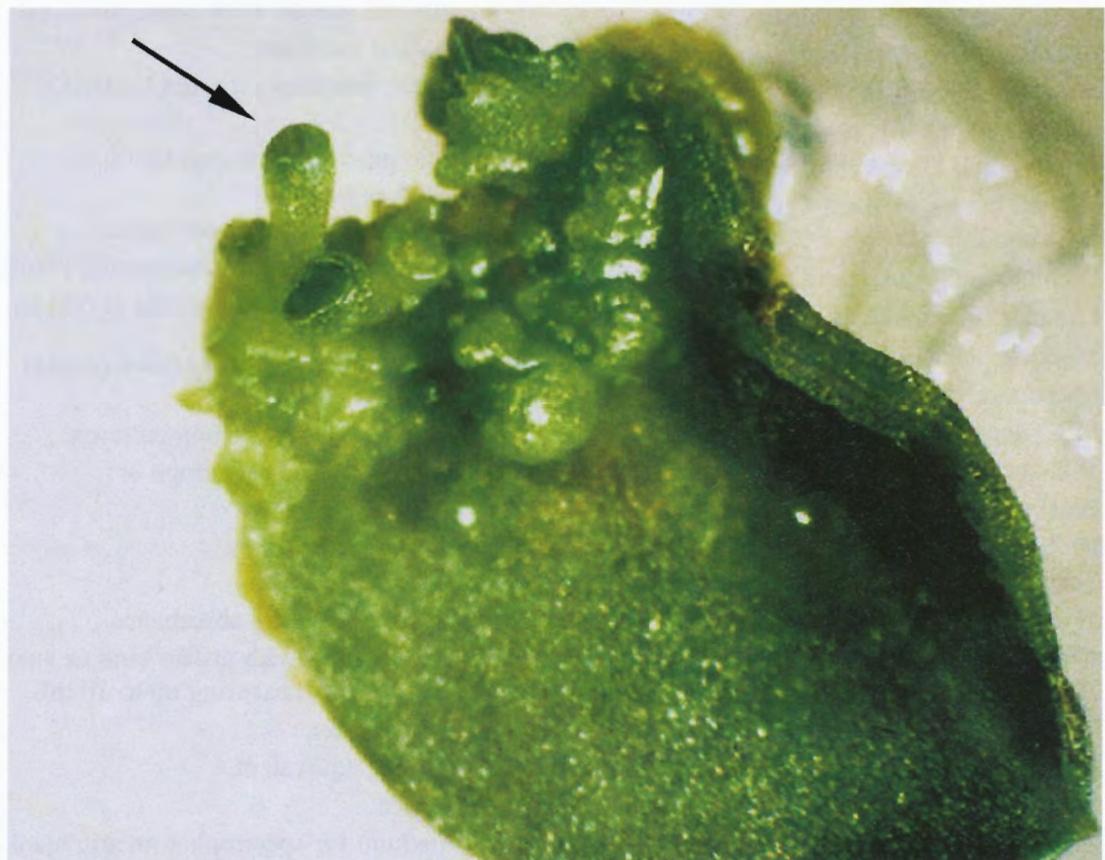
26. Once shoots have developed (1–5 cm) they can be excised by cutting away any root-like structures or callus and placing them on MSOR medium in tissue culture vessels (Figure 7). Continue growth under a 16 hours photo period at 28°C.

See page 15 for further information regarding rooting of shoots.

27. Once roots have formed, transfer plantlets to larger containers of MSO medium to allow shoots to develop and the plants to elongate. Grow under a 16 hour photo period at 28°C. (Figures 8, 9). Plants can be multiplied through nodal cuttings.

### Transfer to soil and acclimatisation

28. Plantlets are transferred to soil and acclimatised as described for plantlets regenerated from somatic embryos (see page 16)



**Figure 14.** Formation of shoot primordia at the cut edge of a peanut leaflet after ~3 weeks in tissue culture.  
(Courtesy T. Newton)

## Reagents and Equipment

### Establishing an *A. tumefaciens* culture

4°C storage facility such as a refrigerator.

28°C incubator.

Glycerol stock or culture plate of *Agrobacterium tumefaciens* carrying plasmid of interest.

YEB agar plate(s) containing appropriate antibiotics.

### Day 1: Sterilisation and sowing of peanut seed (for 60 seed)

0.5 L non-sterile beaker.

0.5 L sterile beaker.

70% ethanol for use in laminar flow hood.

2–2.5 L sterile distilled water.

Absolute alcohol for sterilising tools (i.e. if not using Bacti-Cinerator).

At least 60 healthy, mature peanut seeds.

12.5% stock bleach/chlorine.

Bunsen burner or Bacti-Cinerator in laminar flow hood.

Dichloroisocyanuric acid powder (DICA).

Laminar flow hood.

Marking pen.

Micropore™ tape.

MSO agar plates.

Non-sterile tissue culture jar.

Plant growth chamber at 28°C with 16 hour photo period.

Scalpel blade and other tissue culture tools such as forceps.

Sterile 250 mL glass bottle or equivalent.

Sterile blotting paper (e.g. 3MM or paper towels).

Sterile petri dishes.

Sterile strainers.

Sterile tissue culture jar.

Tabletop shaker.

Tween-20.

### Day 1: Starting *Agrobacterium* culture

3–4 × 10 mL.

16-streak plate with single colonies of *A. tumefaciens* carrying plasmid of interest.

28°C shaking incubator.

Appropriate antibiotics for agrobacterial growth.

Sterile inoculation loop.

### Day 2: Setting up *Agrobacterium* culture for inoculation of explants

3–4 starter cultures of *A. tumefaciens* carrying the plasmid of interest.

3–4 × 100 mL sterile YEB liquid medium.

28°C shaking incubator.

Appropriate antibiotics for agrobacterial growth.

Automatic pipette for measuring up to 200 µL and sterile tips.

Cuvettes for measuring absorbance.

Spare medium for spectrophotometric blank.

Spectrophotometer for measuring at 600 nm.

### Day 3: Preparing the *Agrobacterium* culture for inoculation

3–4 overnight *Agrobacterium* cultures.

Beckman high-speed centrifuge or equivalent.

Cold MS3a medium.

Cold MS3a/AS medium.

Cuvettes for measuring absorbance.

Graduated pipettes with griffin bulb or automatic pipettes for measuring up to 10 mL.

Ice and ice bucket.

JA20 rotor or equivalent.

Marking pen.

Spare medium for spectrophotometric blank.

Spectrophotometer reading at 600 nm.

Sterile blotting paper such as Whatman #1.

Sterile Oakridge tubes or equivalent for JA20 rotor.	MS3aCef <sub>300</sub> agar plates.
Vortex.	Plant growth chamber at 28°C with 16 hour photo period.
<b>Day 3: Preparation and inoculation of explants</b>	Plates of peanut leaflet explants co-cultivating with <i>Agrobacterium</i> .
70% ethanol for use in laminar flow hood.	Sterile scalpel blades with handles, sterile forceps etc.
2 day old sterile germinated peanut seeds (at least 60).	
5–6 × MS3a/AS agar plates.	
Absolute Bacti-Cinerator).	
Bunsen burner or Bacti-Cinerator in laminar flow hood.	
Laminar flow hood.	
Marking pen.	
Micropore™ tape.	
Non-sterile container such as a beaker.	
Overnight culture of <i>A. tumefaciens</i> resuspended in MS3a/AS.	
Parafilm.	
PECA (optional, see below).	
Plant growth chamber at 24°C with 16 hour photo period.	
Sterile blotting paper such as Whatman #1.	
Sterile petri dishes (bacteriological) or equivalent.	
Sterile scalpel blades with handles, sterile forceps etc.	
Tabletop shaker.	
<b>Day 5: Recovery of explants from co-cultivation</b>	
70% ethanol for use in laminar flow hood.	
Absolute alcohol for sterilising tools (i.e. if not using Bacti-Cinerator).	
Bunsen burner or Bacti-Cinerator in laminar flow hood.	
Laminar flow hood.	
Marking pen.	
Materials for assaying transient gene expression eg GUS histochemical stain.	
Micropore™ tape.	
	<b>Day 9–10 and Onwards: Selection, regeneration and rooting</b>
	70% ethanol for use in laminar flow hood.
	Absolute alcohol for sterilising tools (i.e. if not using Bacti-Cinerator).
	Bunsen burner or Bacti-Cinerator in laminar flow hood.
	Dissecting microscope for observing cultures.
	Laminar flow hood.
	Marking pen
	Micropore™ tape.
	MSB5 Cef <sub>300</sub> H <sub>20</sub> agar plates.
	Plant growth chamber at 28°C with 16 hour photo period.
	Plates of peanut leaflet explants recovering from co-cultivation.
	Sterile scalpel blades with handles, sterile forceps etc.
	<b>Transfer to soil and acclimatisation</b>
	70% ethanol for use in laminar flow hood.
	Absolute alcohol for sterilising tools (i.e. if not using Bacti-Cinerator).
	Bunsen burner or Bacti-Cinerator in laminar flow hood.
	Laminar flow hood.
	Marking pen.
	Micropore™ tape.
	MSOR medium in tissue culture vessels.
	Plant growth chamber at 28°C with 16 hour photo period.
	Plates with regenerating peanut shoots.
	Sterile scalpel blades with handles, sterile forceps etc.

## **(B) Transformation using somatic embryos as explants, regeneration via embryogenesis and organogenesis**

We have compared different ages of embryogenic tissue as explants for *Agrobacterium*-mediated transformation. Zygotic embryos used immediately or cultured for 6 and 12 days on embryogenesis medium were inoculated with *Agrobacterium* and further cultured on the same embryogenesis medium. No transformed secondary somatic embryos developed. However, inoculation of somatic embryos that were formed following 7 weeks on embryogenesis medium and then cultured for secondary embryogenesis resulted in transgenic embryos. Thus, we have shown that somatic embryos are a suitable target for transformation using *Agrobacterium tumefaciens* (ACIAR PN 9439 Annual Report, 1998).

Somatic embryo explants are developed as described for 'microprojectile bombardment of peanut embryos' and are inoculated as described for '*Agrobacterium*-mediated transformation of peanut leaflets'. Transgenic somatic embryos are thus generated via embryogenesis on hygromycin B-containing selective medium and then transferred to medium without hygromycin B but with high cytokinin for plantlet regeneration via organogenesis.

This method of transformation is not as efficient as microprojectile bombardment of somatic embryos. In experiments comparing the two methods side by side, 1.8% of inoculated explants gave rise to transformed tissue compared with 4.5% of bombarded tissue. However, if you do not have access to a gene gun, this method would be a useful means by which to develop transgenic peanut, since from 1000 embryos you can expect to regenerate 18 transgenic embryos from which you can regenerate plants. Of the regenerating embryos, 100% were shown to carry the gene of interest.

### **Timetable**

#### **Preparation of explants:**

- Week 1: Sterilisation of zygotic embryos and initiation of embryogenic callus.
- Weeks 2–6: Development of embryogenic callus.
- Weeks 7–12: Maintenance and proliferation of embryogenic callus.

Inoculation of explants can be done at weeks 7–12. We recommend laying down larger numbers of explants at the initiation stage to ensure as many embryos as possible are available for inoculating at approximately week 7, otherwise the numbers can be bulked up by culturing until week 12. As much as possible, aim for an overall reduced time in culture.

- Day 1: Starting *Agrobacterium* culture.
- Day 2: Setting up *Agrobacterium* culture for inoculation of explants.
- Day 3: Inoculation of explants.
- Day 5: Recovery of explants from co-cultivation.
- Day 9–10 and onwards:
  - Selection, regeneration and rooting of transformed plantlets.
  - Transfer to soil and acclimatisation of transformed plants.

## **Experimental Design**

A typical experiment would include the following:

Explants are embryogenic callus pieces, which have been cultured for 7–12 weeks prior to inoculation with *Agrobacterium*. The reasoning for each treatment is described for *Agrobacterium*-mediated transformation of leaflets.

- (1) Explants infected with *Agrobacterium* carrying the binary plasmid of interest and placed onto selective medium.
- (2) Explants infected with *Agrobacterium* carrying the plasmid of interest and placed onto non-selective medium.
- (3) Explants not infected with *Agrobacterium* and placed onto selective medium.
- (4) Explants not infected with *Agrobacterium* and placed onto non-selective medium.
- (5) An extra control that can be included but is usually left out is explants infected with *Agrobacterium* carrying a plasmid which is only the vector base of the plasmid used in (1)

## **Procedure**

### **Week 1: Sterilisation of zygotic embryos**

1. Zygotic embryos are sterilised as described on **page 7**.

### **Weeks 2–12: Initiation and maintenance of embryogenic callus**

2. Somatic embryo cultures are initiated and maintained as described on **page 8–9**.

### **Week 12, Day 1: Starting *Agrobacterium* culture**

3. The *Agrobacterium* culture is started as described on page 26.

### **Week 12, Day 2: Setting up *Agrobacterium* culture for inoculation of explants**

4. The *Agrobacterium* culture is set up for inoculation as described on page 26.

### **Week 12, Day 3: Preparing the *Agrobacterium* culture for inoculation**

5. The *Agrobacterium* culture is prepared for inoculation as described above. However, rather than washing and finally resuspending the bacterial pellets in MS3a/AS, use MS3PG/AS instead.

### **Week 12, Day 3: Preparation and inoculation of explants**

6. Transfer the somatic embryos to a screw-capped container and pour in the agrobacterial culture.

Place on a tabletop shaker for 20 minutes at RT.

Transfer the inoculated embryos to agar plates containing MS3PG/AS.

Label and seal with Micropore™ tape and place in the dark at 24°C for 2 days.

Somatic embryos are larger than leaflets and therefore easier to handle, thus a PECA will not be needed. Co-cultivate the embryos in the dark so that they do not turn green.

### **Week 12, Day 5: Recovery of explants from co-cultivation**

7. Following co-cultivation, transfer explants to MS3PGCef<sub>300</sub> plates to recover and to kill off the *Agrobacterium*. Explants can be placed in any orientation with up to ~30 explants per plate.

Plates are labelled and sealed with Micropore™ tape before being placed into a plant growth chamber at 28°C in the dark for 45 days to recover.

### **Day 9–10 and Onwards: Selection, regeneration and rooting**

8. Selection, regeneration and rooting of transgenic peanut plantlets is done as described on page 30. Transgenic tissue is selected on MS3PG/Cef<sub>300</sub>/H<sub>2</sub>O and rooted on MSOR/Cef<sub>300</sub> (i.e. MSOR containing 300 mg/L cefotaxime) to ensure the *Agrobacterium* is killed off.

### **Transfer to soil and acclimatisation**

9. Plants are transferred to soil and the glasshouse as described on page 16.

## **Reagents and Equipment**

### **Week 1: Sterilisation of zygotic embryos**

As described on page 20.

### **Weeks 2–7/12: Initiation and maintenance of embryogenic callus**

As described on page 20.

### **Week 7/12, Day 1: Starting *Agrobacterium* culture**

As described on page 32.

### **Week 7/12, Day 2: Setting up *Agrobacterium* culture for inoculation of explants**

As described on page 32.

### **Week 7/12, Day 3: Preparing the *Agrobacterium* culture for inoculation**

As described above, except use liquid MS3PG/AS, not MS3a/AS.

## **Week 7/12, Day 3: Preparation and inoculation of explants**

As described on page 33, except:

Explants are somatic embryos

Co-cultivation on MS3PG/AS, not MS3a/AS

Plant growth chamber, dark at 24°C

## **Week 7/12, Day 5: Recovery of explants from co-cultivation**

As described on page 33. Use MS3PGCef<sub>300</sub> not MS3a/Cef<sub>300</sub>

Plant growth chamber, in the dark at 24°C

## **Day 9–10 and Onwards: Selection, regeneration and rooting**

As described on **page 33**.

except: Use MS3PG/Cef300/H<sub>2</sub>O for selecting and regenerating embryos

Use MSOR/Cef300 for rooting shoots

## **Transfer to soil and acclimatisation**

As described on **pages 21 and 33**.

## **2.3 Recipes**

### **General Notes**

Where appropriate, all autoclaving is done at 121°C, 120 psi for 20 minutes.

Sterile solutions are handled in a laminar flow hood.

### **Antibiotics**

All antibiotics are potentially hazardous to one's health. Therefore, they should be treated with extreme care. Weigh out wearing a face mask, gloves and a lab coat. i.e. avoid ingesting powder. In general, it is easiest to prepare antibiotics as 1000 × stocks.

#### **300 mg/mL cefotaxime**

Cefotaxime is also known by the commercial name Claforan (Hoechst Marion Russel). We have generally received cefotaxime as a sterile powder in sterile vacutainers. If you do not receive it in this format, proceed as for any other powder.

Using a sterile needle and syringe, inject 6.7 mL of sterile distilled water into 1 vacutainer vial of cefotaxime containing 2 g of powder.

Mix thoroughly to dissolve.

Using a sterile syringe and needle, remove liquid from vial and aliquot into sterile Eppendorf tubes.

Alternatively, remove rubber bung from the vial of cefotaxime and add 6.7 mL of distilled water.

Filter sterilise through a 0.2 µm sterile filter into a sterile container and aliquot into sterile Eppendorf tubes (the solution can be filtered directly into sterile tubes).  
Store frozen at -20°C.

## **20 mg/mL hygromycin B**

An alternative to making this solution up yourself is to buy it ready-made sterile from a supply company such as Sigma or Life Technologies. Shop around as the prices and concentrations can vary enormously. Purchased stocks should also be aliquoted and frozen for storage. If the concentration you buy is not 20 mg/mL, adjust the volume you add to the media accordingly.

Dissolve 200 mg of hygromycin B in 10 mL of distilled water.  
Filter-sterilise through a 0.2 µm filter into a sterile container.  
Aliquot in 1 mL aliquots in sterile Eppendorf tubes.  
Store frozen at -20°C.

## **50 mg/mL kanamycin**

Dissolve 500 mg of kanamycin in 10 mL of distilled water.  
Filter sterilise through a 0.2 µm filter into a sterile container.  
Aliquot into 1 mL aliquots in sterile Eppendorf tubes.  
Store frozen at -20°C.

## **20 mg/mL rifampicin**

Rifampicin is toxic, handle with extra care.

Dissolve 200 mg of rifampicin in 10 mL of methanol. Filter sterilise through a 0.2 µm filter into a sterile container.  
Aliquot into 1 mL aliquots in sterile Eppendorf tubes.  
Store frozen and in the dark at -20°C.

# **Plant Growth Hormones**

## **1 mg/mL 6-benzyl adenine (BA)/6-benzyl amino purine (BAP)**

Dissolve 250 mg of dry BAP powder in 1 mL 1 M KOH.  
Add 249 mL distilled water.  
Filter sterilise through a 0.2 µm filter into a sterile container.  
Store the solution in the dark at 4°C.  
Do not keep for longer than 6 months.

While the pH of these solutions is not tested prior to storage, the pH of the plant growth media should be tested each time it is made and should be 5.7–5.8.

## **1 mg/mL naphthalene acetic acid (NAA)**

Dissolve 250 mg of dry NAA powder in 1 mL 1 M KOH.

Add 249 mL distilled water.

Filter sterilise through a 0.2 µm filter into a sterile container.

Store the solution in the dark at 4°C.

Do not keep for longer than 6 months.

While the pH of these solutions is not tested prior to storage, the pH of the plant growth media should be tested each time it is made and should be 5.7–5.8.

## **Bacterial Growth Media**

### **YEB medium**

5 g/L beef extract (Gibco/Life Technologies).

1 g/L yeast extract (Gibco/Life Technologies).

5 g/L peptone #190 (Gibco/Life Technologies).

5 g/L sucrose.

0.5 g/L MgSO<sub>4</sub>.7H<sub>2</sub>O.

Dissolve the above in 800 mL distilled water.

Adjust pH to 7.2 with either NaOH or HCl.

Make up to 1 L with distilled water.

For solid medium add 15 g agar per litre before autoclaving.

Sterilise by autoclaving.

Store at room temperature until opened and then at 4°C.

For use, carefully melt in a microwave or over a Bunsen burner. When cooled to hand temperature, add appropriate antibiotics, mix and pour into sterile petri dishes. Store petri dishes at 4°C.

## **Plant Growth Media**

All of the recipes below are based on the use of MS Basal Salts powder (Gibco/Life Technologies) and ready-made vitamins solution (SIGMA). The vitamin solution includes myo-inositol. Gibco/Life Technologies also offers a product with the salts and vitamins combined. Either use purchased salts and vitamins or prepare your own according to the instruction of Murashige and Skoog (1962). If the vitamin solution you purchase is a high concentration i.e. 1000×, you may find it crystallises on storage at 4°C. You can dilute it down to 100× with sterile distilled water if desired. We recommend aliquoting this solution into smaller volumes to prevent the chance of the stock solution becoming contaminated.

Following autoclaving, all media should be stored at room temperature (i.e. 25°C) in the dark (bottle can be wrapped in aluminium foil) and should not be kept for more than 4 weeks, especially the media containing auxins. Medium containing hygromycin B should not be stored for more than 2–3 weeks.

**Table 1.** Media formulations for the regeneration of transgenic peanut via embryogenesis #.

	MSO	MS5PG	MS3PG	MS1PG	MS3PGH <sub>20</sub>	MS3PGO	MSC	MS10	MSOR
MS basal salts	4.33 g/L	4.33 g/L	4.33 g/L	4.33 g/L	4.33 g/L				
100 × MS vitamins	10 mL/L	10 mL/L	10 mL/L	10 mL/L	10 mL/L				
Sucrose	20 g/L	30 g/L	30 g/L	30 g/L	30 g/L	30 g/L	20 g/L	20 g/L	20 g/L
Glutamine		1 g/L	1 g/L	1 g/L	1 g/L	1 g/L			
Picloram		5 g/L	3 g/L	1 g/L	3 g/L	3 g/L			
20 mg/mL hygromycin B					1 mL/L				
D-sorbitol						0.2 M			
D-mannitol						0.2 M			
Charcoal							1 g/L		
1 mg/mL BAP								10 mL/L	
1 mg/mL NAA									1 mL/L
Agar*	8 g/L	8 g/L	8 g/L	8 g/L	8 g/L				

# Media are prepared by adding all of the components except the antibiotics and adjusting the pH to 5.7 prior to autoclaving. It is important to maintain this method of media preparation to ensure the concentrations of the various media components are correct. All antibiotics are added after autoclaving, otherwise they will be rendered ineffective.

\* Agar added only if solid medium required.

**Table 2.** Media formulations for the regeneration of transgenic peanut via organogenesis #.

	MS3a	MS3a/AS	MS3aCef <sub>300</sub>	MS3aCef <sub>300</sub> H <sub>20</sub>	MSB5 Cef <sub>300</sub> H <sub>20</sub>
MS basal salts	4.33 g/L	4.33 g/L	4.33 g/L	4.33 g/L	4.33 g/L
100× MS vitamins	10 mL/L	10 mL/L	10 mL/L	10 mL/L	10 mL/L
Sucrose	20 g/L	20 g/L	20 g/L	20 g/L	20 g/L
1 mg/mL BAP	3 mL/L	3 mL/L	3 mL/L	3 mL/L	5 mL/L
1 mg/mL NAA	1 mL/L	1 mL/L	1 mL/L	1 mL/L	
100 mM acetosyringone		1 mL/L			
300 mg/mL cefotaxime			1 mL/L	1 mL/L	1 mL/L
20 mg/mL hygromycin B				1 mL/L	1 mL/L
Agar*	8 g/L	8 g/L	8 g/L	8 g/L	8 g/L

# Media are prepared by adding all of the components except the growth hormones and antibiotics prior to autoclaving. That is, the BAP, NAA, cefotaxime and hygromycin are added after autoclaving.

\* agar added only if solid medium required.

## Miscellaneous

### 0.1 M Spermidine

Under sterile conditions, dissolve 1 g of spermidine powder in 6.9 mL of sterile distilled water (this gives a 1M stock).

Aliquot into 1 mL portions and store at -80°C.

Dilute a 1 mL aliquot 10-fold to get 10 × 1 mL aliquots of a 0.1 M solution and store these at -80°C.

Once you have thawed an aliquot, do not refreeze and reuse, i.e. discard after use.

Spermidine is highly hygroscopic i.e. it absorbs water from the atmosphere. Therefore, once you have purchased a bottle of the powder, you will need to make up this solution using the entire contents of the bottle to ensure the final concentration is correct. Depending on how many shots you do at a time, you may wish to aliquot the 0.1 M stock into aliquots smaller than 1 mL.

### 2.5 M CaCl<sub>2</sub>

Dissolve 36.8 g of CaCl<sub>2</sub>.2H<sub>2</sub>O in 100 mL of distilled water.

Autoclave to sterilise.

Under sterile conditions, aliquot into smaller volumes.

Store at RT.

### GUS assay buffer and staining for GUS activity

This is a histochemical stain that detects the expression of the *uidA* gene encoding β-glucuronidase (GUS). It uses the substrate 5-bromo-4-chloro-3-indolyl-B-D-glucuronide, also known as X-Gluc.

For 20 mL add together:

4 mL 1 M NaHPO<sub>4</sub> (pH 7.0).

1 mL 0.1 M potassium ferrocyanide.

1 mL 0.1 M potassium ferricyanide.

120 µL Triton-X-100.

13.83 mL sterile distilled water.

Weigh out 30 µg X-Gluc and dissolve in 50 µL DMSO.

Add this to the above mixture, mix and add to plant tissue.

Incubate overnight at 37°C

This buffer can be made and stored at -20°C but repeated thawing and freezing is not recommended. It is easy to make it up from stock solutions when required. It can also be aliquoted if 20 mL is too large a volume for your tissue. If you are concerned about the substrate penetration into the plant tissue, you can vacuum infiltrate the tissue/buffer mix for about 2 minutes at RT before incubating at 37°C.

# CHAPTER 3

## Analysis of Transgenic Peanut Lines

### 3.1 Initial screen for transgenic tissues: PCR

POLYMERASE chain reaction (PCR) is a powerful technique to detect the presence of specific sequences in plant samples. It is both sensitive and rapid, and will allow the large-scale initial screening of putative transformed tissue for the presence of the transgene. We have used PCR to detect single and multiple copy transgenes in transformed regenerating peanut embryos and in the leaves of transgenic peanut plantlets growing in tissue culture or in the glasshouse. Oligonucleotide primers may target any area in the introduced gene, e.g. promoter, terminator, gene of interest, reporter gene, selectable marker gene or flanking regions. For PCR, DNA primers are used, short oligonucleotides, specific for the gene of interest. A PCR assay with these primers, even using relatively crude DNA as the template, will allow exponential amplification of the sequence between the primer binding sites. PCR should only be used as an initial, quick screen and does not replace Southern blot analysis to demonstrate integration into the peanut genome and approximate number of integrated copies. Care has to be taken when using PCR on tissue transformed with *Agrobacterium* to ensure that no traces of residual *Agrobacterium* persist. PCR cannot distinguish between the target sequence present in the bacterial plasmid or in the plant genome and will amplify both, potentially leading to false positive results.

There have been many methods published describing the analysis of transgenic plants using PCR. We have shown the method outlined below to be the simplest of methods to detect many different genes from a variety of tissue types of several different species (Higgins et al. unpublished). Importantly, it has also been successfully applied by different researchers demonstrating that it is a procedure adaptable to different laboratories.

This method is very simple. A small amount of tissue is soaked in template preparation solution (TPS) and heated to release the cellular contents, including the DNA. The tissue debris is then separated by centrifugation from the DNA-containing liquid, which is included in a PCR. This method has been used to amplify products up to 1500 bp in length. However, it is important to note the conditions under which you may observe a false negative result. You need to be aware of the following factors:

1. The ratio of the TPS volume to the amount of tissue can affect the amplification. When too little TPS is used relative to the amount of tissue, the level of PCR inhibitors (present in peanut tissues) can be high, even when the sample is diluted in water. This may be particularly noticeable, if you are trying to amplify a large fragment. We occasionally encounter false negatives diluting 2 mm<sup>2</sup> peanut tissue 1:10. The safest way is to measure the OD<sub>260</sub> in a spectrophotometer and to adjust the DNA concentration in the TPS extract to 50–100 ng/μL sample for PCR (Sudarsono, pers. comm.).
2. The pH of the TPS can influence the success of the PCR. We found that by increasing the pH from 7.4 to 8.0 for peanut we obtained more predictable results.

If amplification is unsuccessful, we recommend reducing the amount of tissue being tested, increasing the volume of TPS or diluting the sample further than 1:10 prior to PCR.

Inclusion of primers for amplification of an internal multiple gene copy standard would also identify false negative reactions. The procedure we have outlined below has proven consistent for the PCR amplification of transgenes of up to 1500 bp in length from both peanut embryo and leaf tissue.

## Leaf soak PCR

1. Remove a tissue sample of approximately 2 mm<sup>2</sup> from the plant(s) of interest or a whole somatic embryo from a culture plate.  
Place into a sterile Eppendorf tube.

The tissue can be either from a fresh plant or frozen tissue. The tissue can be either leaf or somatic embryos.

2. Add 50 µL of Template Preparation Solution (TPS) (100 mM Tris-HCl pH 8.0, 1 M KCl, 10 mM EDTA) to leaf tissue, 30 µL of TPS per somatic embryo.  
Grind the tissue briefly using a pestle suitable for microfuge tubes.  
Heat at 95°C for 10 minutes.  
Place on ice for 2 minutes.  
Centrifuge briefly to sediment any solid matter.

Glass or metal pestles designed for use with Eppendorf tubes can be purchased or prepared by your workshop. Plastic pestles can also be purchased together with specialist 1.5 mL tubes. While these pestles are designed to be disposable, we have used them several times by cleaning and autoclaving in-between uses.

3. Remove a 1 µl sample (diluted 1:10 in sterile distilled water) and add to the following mixture for a final volume of 25 µL:

10 mM Tris-HCl pH 8.3.  
50 mM KCl.  
1.5 mM MgCl<sub>2</sub>.  
50 µM dNTPs.

} 1 × PCR reaction buffer

100 nM primer A.  
100 nM primer B.  
1.5 U *Taq* DNA polymerase.

sterile distilled water to 25 µL

If you are using a PCR machine that does not have a 'hot top', then add 30 µL of mineral oil to the tube.

In some cases, the TPS extract may inhibit the PCR, if undiluted. We believe that this is due to the presence of phenolic compounds within the crude plant extract as well as the high KCl concentration. It will probably be necessary to dilute the extract before adding to the reaction mixture. We have recommended a 1:10 dilution in sterile distilled water. If you still do not detect a PCR product, try diluting the extract further than 1:10.

Most companies that sell heat-stable DNA polymerase, also provide the buffer to go with it. We have used *Taq* from Roche Diagnostics (formerly Boehringer Mannheim). They provide a 10 × buffer that

has Mg<sup>2+</sup> in it at the recommended concentration as well as other buffer components for PCR. If you use this buffer, you can add it instead of the Tris.HCl/KCl/ MgCl<sub>2</sub> by diluting it to a final concentration of 1×. Check the buffer you have to see if it provides all the components listed above at the correct concentrations. If not, either use what you have ensuring the Mg<sup>2+</sup> concentration is correct or make it up yourself.

Depending on the type of PCR machine you have, you will use either a 200 or 500 µL tube to set up the PCR. The reaction outlined above utilises *Taq* DNA polymerase, however, there are a myriad of heat stable polymerases on the market now. We expect that other polymerase will also work fine under these reaction conditions, but check the manufacturer's information.

4. Carry out the PCR as follows:

Denaturation	1 cycle	94°C for 3 minutes.
Amplification	35 cycles	94°C for 45 secs. 50–60°C for 45 secs. 72°C for 90 secs.
Final extension	1 cycle	72°C for 10 minutes.
Soak		4–15°C until removal from machine.

We have carried out PCR under these conditions using PE Applied Biosystems GeneAmp PCR System 480 or 9700 **DNA** thermal cyclers. While it is possible that different PCR machines may contribute to the success of this PCR, we generally do not expect that to be an important factor. The most likely causes of failure will either be the presence of inhibitors in the TPS extract, or the sub-optimal design or annealing temperature of the primers. Depending on the primers that you use, the annealing temperature may need to be adjusted. As a rule of thumb, estimate the melting temperature (T<sub>m</sub>) for your primers using the formula T<sub>m</sub>~ 4 (G+C) + 2 (A+T), and set the annealing temperature to be T<sub>m</sub>-5°C.

5. Analyse and record the PCR products by agarose gel electrophoresis according to the method outlined on **page 50**. Analyse 10 µL of the PCR product.

The percentage agarose you use to form the gel for the analysis of the PCR products depends on the size of the products you are expecting. For products of 0.5–4 kb in length, a 1% gel is appropriate. For products smaller than 0.5 kb, use a 1.5–2% agarose gel. Also use appropriate DNA molecular weight markers.

### Total nucleic acid extraction

If you feel that the TPS extract is too crude and you would like to prepare a purer template, you can isolate pure genomic DNA as described on **page 47**. If you wish to prepare somewhat purer DNA than what is provided by the 'leaf-soak' method described above, but do not want to go to the trouble of preparing ultra-pure DNA then use the alternative protocol outlined below (Smith et al. 1992). We have used this method, as well as TPS extractions for the preparation of template DNA from peanut for use in PCR.

1. Remove approximately 100 mg of tissue from the plant to be analysed and freeze immediately in liquid nitrogen.
2. Transfer the tissue to a sterile Eppendorf tube.  
Grind the tissue to a powder with a pestle suitable for microfuge tubes.

3. Add 5 volumes (i.e. 500 µL) of 0.05 M sodium phosphate buffer, pH 8.0.  
Centrifuge for 5 minutes at 12000 × g at RT.
4. Working in a fume hood, transfer the supernatant to a fresh sterile Eppendorf tube.  
Extract with phenol/chloroform by adding an equal volume (500 µL) of phenol (saturated with 0.1 M Tris.HCl pH 8.0):chloroform:isoamyl alcohol (25:24:1).  
Centrifuge at 12000 × g for 5 minutes at RT.  
Working in a fume hood, transfer upper aqueous phase to a fresh, sterile Eppendorf tube.
5. Working in a fume hood (there will still be traces of the organic solvent), precipitate the DNA by adding 0.1 volume (50 µL) of 2.5 M sodium acetate pH 5.2 and 2 volumes (1000 µL) of absolute ethanol.  
Chill at -70°C for 15–20 minutes or -20°C for at least 2 hours.  
Centrifuge in a microfuge at 12 000 × g for 10 minutes at RT.  
Pour off the alcohol ensuring the pellet is not disturbed.  
Add 500 µL of 70% ethanol.  
Re-centrifuge at 12 000 × g for 5 minutes at RT.  
Pour off ethanol ensuring pellet is not disturbed.  
Centrifuge briefly again (~30 seconds) to bring the liquid to the bottom of the tube, pipette this liquid out without disturbing the pellet.  
Air dry 1–2 minutes.  
Resuspend in 100 µL of sterile distilled water.  
Use 1 µL in a PCR as described in the ‘leaf soak PCR’ section above.

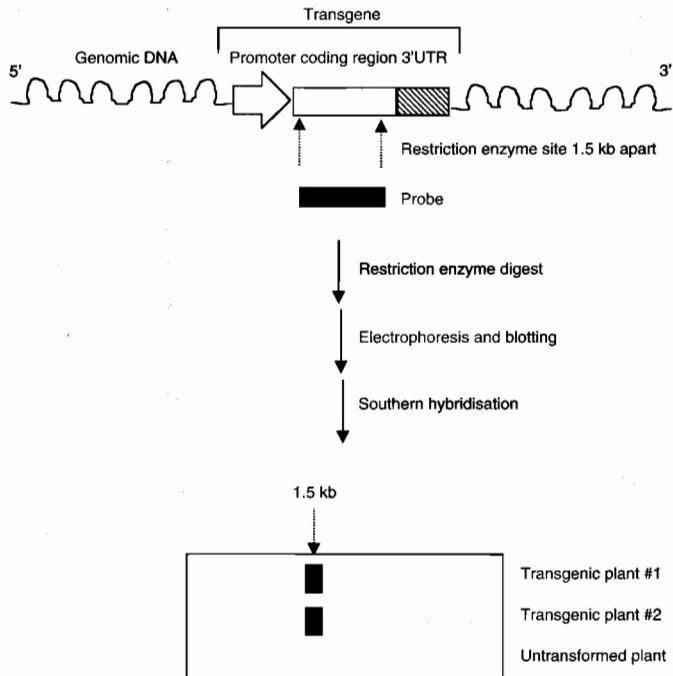
### 3.2 Transgene integration: Southern blot

Once putative transgenic plants have been regenerated, it is important to confirm the presence of the transgene(s) in the plant(s). More importantly perhaps is confirming the integration of the transgene in the genomic DNA of the plant. It is also essential to ensure that the transgene is passed on through the germline to the progeny. Thus genomic DNA analysis of generations (i.e. T<sub>1</sub>, T<sub>2</sub> etc.) beyond the primary transformant (the T<sub>0</sub> generation) is also important. For breeding purposes, it is essential to know how many copies of the transgene are present and if there are any rearranged or incomplete copies.

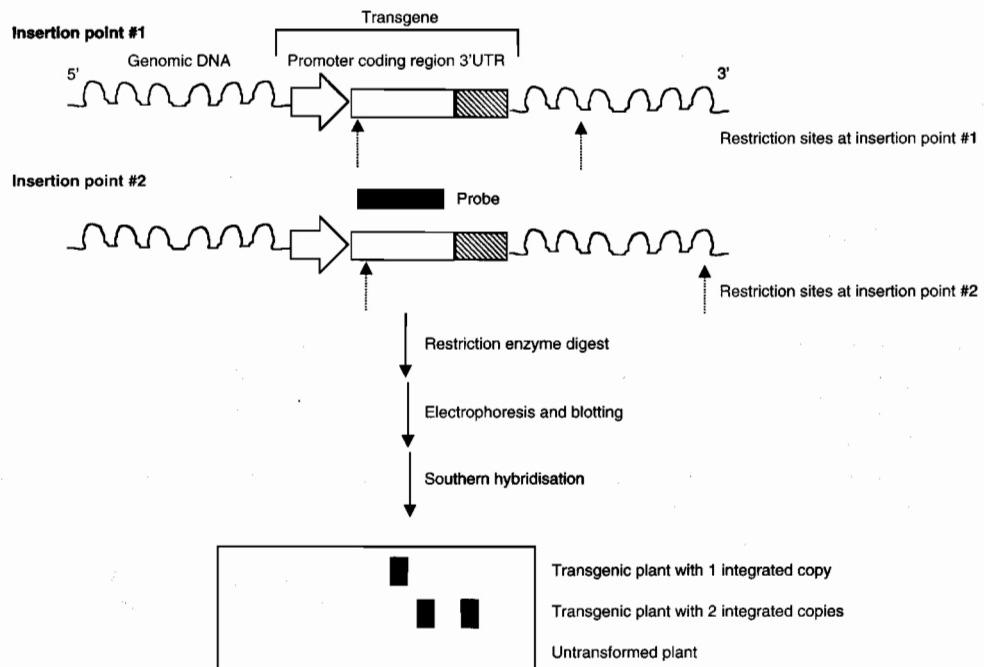
The presence of a transgene can be detected by either PCR (Chapter 3.1) or Southern hybridisation. PCR only determines if the transgene is present or not; it does not provide confirmation of the integration of the transgene. The presence *and* integration of the transgene can be confirmed by Southern hybridisation of restriction enzyme digested genomic DNA with a probe specific for either all or a portion of the transgene(s). Genomic DNA is extracted, then it is digested into smaller pieces with a specific restriction enzyme. The restriction fragments are then separated through an agarose gel by electrophoresis and then transferred to a nylon membrane. The DNA held on the membrane is then hybridised with a probe specific for the transgene that is labelled with either a radioisotope or with a non-radioactive label. This hybridisation is then detected by the appropriate method for the label used.

The presence of the transgene is shown by digesting the DNA with a restriction enzyme that will cut out the transgene sequence from the genomic DNA (Figure 15A). The actual integration of the transgene and an estimate of the transgene copy number are also confirmed

(A)



(B)



**Figure 15.** Southern blot schematic diagrams for the detection of a PStV CP transgene in the genome of transformed peanut plants. (A) confirmation of the presence of at least one PStV CP transgene; (B) estimation of transgene copy number.

by Southern hybridisation. If a restriction enzyme is used that cuts only once within the targeted transgene sequence, then the hybridisation of a probe specific for that gene will confirm integration and/or rearrangements of the transgene. The number of fragments observed gives an estimate of the copy number per plant genome (Figure 15B).

While there are many methods for carrying out these types of experiment, all of the methods have many features in common. A lot of mythology has arisen around these methods so that a researcher new to this area may be nervous about which ones to choose. From a technical point of view, these methods are in fact straight forward and with an understanding of the chemistry involved, any person skilled in molecular biology can carry out such experiments with ease. One word of caution we would like to offer is that if you are going to use radioactivity to label your probe, ensure that you are certified for the use of such chemicals and you understand the potential risks and dangers. It would also be useful to set aside a particular area within your laboratory specifically for the use of radioisotopes.

## Southern Hybridisation Analysis

There are many procedures for the extraction of genomic DNA. Which one you choose depends on the type of analysis you wish to carry out. If you want to detect the gene by PCR, a relatively crude preparation is sufficient. If you wish to use restriction digestion and Southern hybridisation analysis you will need to prepare purer DNA, since many restriction enzymes can be inhibited by compounds present in crude plant extracts. Regardless of the analysis you wish to carry out, all methods rely on the disruption of the plant cells to release the contents by grinding. For analyses that require purer genomic nucleic acid preparations, this is followed by the denaturation and removal of proteins, carbohydrates and cell debris usually with phenol/chloroform. This is usually sufficient for PCR. For purer preparations, RNA is removed with RNase and then the genomic DNA is precipitated. The method described here for the preparation of pure genomic DNA has worked for several legume species as well as tobacco and cucurbits. If these do not work satisfactorily, we suggest you experiment with other procedures.

### Extraction of pure genomic DNA

1. Remove up to 1 g of plant tissue and wrap in labelled aluminium foil. Place in liquid nitrogen immediately. The tissue can be extracted straight away or stored frozen at -80°C until required.

Any plant tissue can be used for the extraction of genomic DNA, however, leaf tissue generally gives the best yield of DNA. It is advisable to wear safety glasses while grinding tissue in liquid nitrogen and when using phenol/chloroform.

2. Place tissue into mortar and pour liquid nitrogen into mortar, over tissue.
3. Grind tissue in liquid nitrogen to a fine powder ensuring no large pieces of tissue remain.

Add 3 mL of urea extraction buffer and transfer to a sterile polypropylene tube.

Use 3 mL of extraction buffer per gram of tissue. If you are extracting >1 g of tissue, scale up the amount of buffer accordingly. Increase the amount of phenol/chloroform appropriately (see below). Everything else can stay the same.

4. In a fume hood, add 3.5 mL of phenol/chloroform and shake for a minimum of 15 minutes at RT on either a rotating wheel or shaking table top. An emulsion between the phases must be formed.

If extracting 1 g of tissue, a 15 mL tube is appropriate, if extracting more, use a 50 mL tube. You can begin your extractions and place your samples on a rotating wheel or shaking table top while you are extracting the other samples. Make sure the last sample is extracted for at least 15 minutes. If using a shaking tabletop, also mix samples by hand regularly.

5. Separate the phases by centrifugation at top speed in a bench top centrifuge (~3000 r/min) for 10–15 minutes at 15–20°C.
6. In a fume hood, transfer the upper aqueous phase to a fresh polypropylene tube.
7. In a fume hood, add 500 µL of 4.4 M ammonium acetate, pH 5.2 and mix well.
8. Add an equal volume of isopropanol and mix gently.

At this stage, if there is a lot of genomic DNA, it will generally precipitate out as a mucous blob. If there is not much DNA you may not see a precipitate at this stage. Sometimes, the pellet is not very big and the mixing can break the precipitate up into small stringy pieces.

9. Spool DNA out and resuspend in 500 µL of sterile TE, pH 8.0.  
Transfer the DNA solution to a sterile 1.5 mL Eppendorf tube.

If a mucous blob type of precipitate has formed, it is much easier to dissolve the DNA if this precipitate is pulled or spooled out of the tube. The DNA can be removed from the tube using a hook made from a glass Pasteur pipette. Heat the end of a Pasteur pipette in the flame of a Bunsen burner to create a hooked end. Otherwise, try a sterile tip from an automatic pipette.

If the DNA does not form as an aggregated precipitate but has broken up into smaller stringy pieces, pellet the DNA by centrifugation for about 15 minutes at 12 000  $\times$  g at RT. Pour off the supernatant leaving the pellet behind.

Air-dry and resuspend in 500 µL of TE, pH 8.0.

Transfer the DNA solution to a sterile 1.5 mL Eppendorf tube.

If no DNA precipitates at this stage, place at –20°C overnight or –70°C for 30 minutes. Centrifuge at 12 000  $\times$  g for 15 minutes.

Pour off isopropanol, ensuring pellet remains in the tube.

Air-dry and resuspend in 500 µL of TE, pH 8.0.

Transfer the DNA solution to a sterile 1.5 mL Eppendorf tube.

You must ensure that the DNA is fully resuspended. To help this along, you can heat the DNA at 65°C for about 10–15 minutes and leave overnight at 4°C. It is good to give the tube the occasional gentle flick during each of these treatments to encourage the DNA to dissolve. Do not vortex to avoid shearing the DNA.

**10. When the DNA has dissolved, add 10 µL of 10 mg/ml RNase A.**

Leave at RT for 15 minutes.

In a fume hood, do two rounds of phenol/chloroform extractions: add an equal volume of phenol/chloroform, mix the phases thoroughly, but gently and centrifuge at  $12\ 000\times g$  for 10 min at RT. Transfer the upper aqueous phase to a fresh tube.

Repeat, finally transferring the aqueous phase to a fresh tube.

A mixture of RNase A and RNase T1 will also work well. It is possible to omit the RNase step and subsequent phenol/chloroform extractions. RNA should not inhibit the restriction digestion of the genomic DNA; however, determination of the DNA concentration will not be accurate. If you use a spectrophotometer to measure the DNA concentration, the RNA will also be measured. If you use ethidium bromide staining of DNA in an agarose gel, the RNA will also chelate ethidium bromide and may make the visual estimation of DNA concentration difficult, especially if you compare to a standard that does not contain RNA.

You can measure the DNA as total nucleic acid concentration using a spectrophotometer (see below) and ignoring the presence of RNA. This has been adequate for detecting single copy gene sequences using radioactive probes but is not suitable for digoxigenin (DIG)-labelled probes. DIG-labelled probes are more sensitive to the amount of target DNA on a gel therefore if you are using this type of probe, you need to be more accurate when determining the concentration of the genomic DNA. Also, if you wish to quantify the amount of specific sequence within the genomic DNA, you will need to accurately determine the DNA concentration.

If you use an RNase step, you will notice at the phenol/chloroform extraction stage the interface will be quite substantial. Peanut is a starchy plant; thus it is likely that the interface is mostly starch. The interface may be particularly large from a glasshouse grown plant compared with a tissue culture grown plant. When removing the aqueous phase, avoid removing any of the interface. The presence of starch may interfere with subsequent restriction enzyme digestion. If it does, re-extract the DNA with chloroform, i.e. no phenol and re-precipitate.

**11. Add 100 µL of 4.4 M ammonium acetate and mix gently.**

Add 700 µL of cold isopropanol and mix gently.

Re-spool the DNA as described above.

**12. Wash the precipitate in 70% ethanol. If you have a mucous blob, dip the pellet carefully into ethanol and drain on the side of the vessel. If you have had to centrifuge the precipitate, remove the ethanol from the pellet, add about 1 mL of 70% ethanol to the tube carefully without disturbing the pellet, gently wash the pellet by rolling the tube and pouring the ethanol off.**

Air dry for 1–2 minutes.

Resuspend the DNA in 200 µL of TE, pH 8.0.

**13. Determine the concentration from the  $A_{260}$  measurement.**

Before measuring the  $A_{260}$ , ensure the DNA is fully resuspended as described above. The purity of the DNA can also be determined by calculating the  $A_{260}/A_{280}$  ratio. If it is around 2, then the DNA is pure; if it is less, then it is contaminated with protein. If you find in the next step that the DNA does not digest, it may be that the DNA needs re-extracting with phenol/chloroform to remove contaminating protein, or with ether to remove traces of phenol that may be present. Ether extractions are carried out exactly as for phenol/chloroform extractions except that the aqueous phase containing the DNA is the lower phase.

## Digestion of genomic DNA with restriction enzymes

The choice of restriction enzyme will depend on the cleavage sites present within the transgene. To prove integration of the transgene, you need to choose a restriction enzyme that cuts once only within the transgene sequence.

### 1. Set up the following mixture:

Genomic DNA	10–20 µg.
10 × restriction buffer	5 µL.
0.1 M spermidine	2.5 µL.
Restriction enzyme	4U per µg DNA.
Sterile distilled water	to 50 µL total volume.

Most companies provide restriction enzymes with pre-made buffer either 10× or 5×. If you receive a 5× buffer, then adjust the volume accordingly. If you do not receive the buffer with the enzyme, you can make it up yourself as recommended for the particular enzyme by the company. Ensure the buffer you make up is sterile, since DNases that can break down the DNA may be present in an unsterile solution.

If the DNA is too dilute to make up a reaction of 50 µL, make up a mixture with 10–20 µg of DNA and scale everything else up. When the digest is complete, precipitate the DNA and resuspend in a smaller volume for loading on the gel.

The units of a restriction enzyme are defined as the amount of enzyme it takes to cut 1 µg of phage lambda DNA in 1 hour. You will need more enzyme per µg of genomic DNA than you do for lambda or plasmid DNA, hence 4 U per µg is recommended. Spermidine is included in the mixture to assist the enzyme to cut the high molecular weight DNA.

### 2. Leave overnight at the appropriate temperature for the particular restriction enzyme.

### 3. The next day, precipitate the DNA from the digest if you have had to scale up the reaction:

Add 0.1 volume of 3 M sodium acetate, pH 5.2 and 2–2.5 volumes of cold ( $-20^{\circ}\text{C}$ ) ethanol.

Leave at either  $-70^{\circ}\text{C}$  for 15–20 minutes or  $-20^{\circ}\text{C}$  for a minimum of 2 hours.

Centrifuge the DNA at  $12\,000 \times g$  for 20–30 minutes at RT.

Pour off the supernatant, ensuring the pellet remains in the tube.

Add 1 volume of cold 70% ethanol.

Centrifuge at  $12\,000 \times g$  for 15 minutes at RT.

Pour off the supernatant carefully, ensuring the pellet remains in the tube.

Briefly centrifuge to bring the remaining liquid to the bottom of the tube.

Pipette off this liquid without disturbing the pellet.

Air-dry for 1–2 minutes.

Resuspend in ~20 µL of TE, pH 8.0.

Leave on ice, if you are to load the DNA onto the gel soon, otherwise store at  $-20^{\circ}\text{C}$ .

## Agarose gel electrophoresis

Depending on the size of the wells that you use for your gel (see below), you may have to precipitate the DNA, i.e. the well may not be big enough to hold the entire volume of the digest. If this is not the case, you can load the digest directly. In some cases, you may prefer to do this, since by precipitating you risk losing some of the DNA. We have also had good experience in purifying the DNA digest using a Bresaclean kit ([www.geneworks.com.au](http://www.geneworks.com.au)) to remove the enzyme and salts.

1. Prepare a gel tray before making up the gel mixture. This is done either by taping the ends of the tray or, if the gel system you use is designed for this, inserting the tray into the gel rig. See the instructions for your particular gel system. Insert the comb into the tray. Ensure the bench top you are using for pouring your gel is level.

The width of the wells is an important consideration when doing these experiments. You must ensure the well is big enough to hold the digest without spilling any of the DNA out. If the well is too wide, the hybridisation signal may be less intense than that with a narrower well, since the DNA will be spread over a larger area. If the well is too narrow, the hybridisation signal may appear as a blob rather than a discrete band. It is a question of balance.

2. To prepare 100 mL of a 1% gel, weigh out 1 g of agarose into a sterile flask or beaker Add 100 mL of 1 × electrophoresis buffer (i.e. TAE or TBE) (Sambrook et al. 1989)

Depending on the size of the restriction fragment(s) to be resolved, use either TBE or TAE as the electrophoresis buffer. Either buffer system works well for most circumstances but there are some differences, which you may like to consider. TAE resolves better in the upper end of the usual range of fragment sizes (i.e. 4–23 kb) while TBE resolves better at the lower end of the range (0.5–4 kb). It is a matter of convenience and desired result which one you choose. Also, if TAE gels are electrophoresed too long i.e. >20 hrs, a pH gradient will form between the ends of the gel tank and the DNA may run awry. If you need to electrophorese your gel for longer to resolve particular fragments, you can either use TAE and recirculate the buffer, or use TBE. The buffer you use to prepare the gel may contribute to background during hybridisation. If you do get unexplained background, try changing the buffer system.

3. Heat to boiling in either a microwave or over a Bunsen burner until the agarose has completely melted. Be sure you do not over boil the solution to the point where it spills out of the container or water evaporates.

Cool to a temperature where you can hold the agarose solution reasonably comfortably in your hand but the agarose has not started to solidify.

Add 1  $\mu$ L of 10 mg/mL ethidium bromide to the agarose and mix by swirling, being careful not to create air bubbles.

With one fluid movement, pour the agarose into the prepared gel tray.

Leave at RT to set.

The ethidium bromide intercalates into the DNA molecules allowing visualisation of the DNA under UV transillumination. This compound can either be added into the gel mixture so that the DNA becomes stained as it migrates through the gel, or the gel can be soaked after electrophoresis in buffer containing 0.5  $\mu$ g/mL ethidium bromide. There are reasons for and against both of these approaches, in the end, it is a matter of personal choice.

4. To a 50  $\mu$ L digest add 10  $\mu$ L of 6 × loading dye. If you have precipitated the digest and resuspended in 20  $\mu$ L, add 4  $\mu$ L.

There are many different recipes for loading dye solutions. The purpose of this solution is to make the digest heavier than water so the DNA will sink to the bottom of the well, and also to help you track how far the DNA has progressed through the gel during electrophoresis. The most commonly used dyes are bromophenol blue and xylene cyanol: bromophenol blue runs at ~500bp in a 1% gel. The loading dyes contain either sucrose, ficoll or glycol (Sambrook et al. 1989).

5. Place the set gel into the gel rig, ensuring any tape and the comb have been removed.  
Pour 1 × electrophoresis buffer (TAE or TBE, but the same with which you made the gel) into the gel rig to cover the gel by ~1 cm.  
Load digested DNA into the wells, one lane per digest, ensuring the solution does not spill out.  
If planning to use a DIG-labelled probe, also load DIG-labelled DNA size markers (20–50 ng per lane). If using a radioactive probe, load unlabelled DNA markers (~0.25–0.5 µg per lane).

DIG-labelled size markers will tell you if your detection step has worked later in the procedure.

You may also want to include as a positive control a lane of plasmid DNA carrying the gene(s) that was used to transform the plant. Of this DNA, load only ~10 pg.

Another sample to include is digested genomic DNA from an untransformed plant, which serves as a negative control.

7. Assemble the gel rig and hook it up to a power supply, remembering that DNA is negatively charged at neutral pH and will migrate towards the positive electrode.  
Start the gel electrophoresis by setting the desired voltage.  
Continue until the bromophenol blue is ~1 cm from the bottom of the gel.

The appropriate speed at which to electrophorese a gel is described in Sambrook et al. (1989).

8. Take a photograph of the ethidium bromide-stained gel as a record.  
If you have included unlabelled markers, mark the position of each marker.

This can be done by either using a needle dipped in Indian ink and piercing each band once (wash the excess away) or cutting the edge of the gel at each position. The ink will transfer to the membrane as a faint black spot that allows you to see the positions of the markers. Cutting the gel may cause it to break at a later stage, so take care.

Polaroid photographs have been the method of choice to date for recording electrophoresis results. They are stable over time and provide very good contrast, so that even faint bands are often observable. More recently, digital cameras have emerged as alternatives by capturing video images onto thermal paper or preferably as computer files.

## Southern blotting

Southern blotting was first described by Southern (1975). It refers to the transfer of DNA to a solid matrix, in this case a nylon membrane, where the DNA is fixed in position. The transfer can be done either by capillary blotting as described below or electroblotting in an electric field. Specific sequences within the DNA held on the membrane can be identified by hybridising the membrane with a labelled probe, either DNA or RNA. Prior to the transfer, the DNA being probed must be separated by electrophoresis as described above and then denatured in situ, so that the double-stranded DNA becomes single-stranded. If the DNA is not denatured, the probe cannot bind to the target sequence. Southern blot membranes or filters can be stored for some weeks prior to hybridisation.

1. (Optional) After electrophoresis of DNA through agarose and photography of the gel, transfer the gel to a tray containing 0.25 M HCl at RT.  
Place on a shaking table top and shake gently for 5 minutes. Ensure the gel is covered with liquid, as it may float.  
Pour off the HCl and briefly rinse the gel with distilled water.

This is a depurination step. Higher molecular weight DNA is more difficult to transfer to membranes than lower molecular weight. If the DNA is treated briefly with dilute HCl, this depurinates the DNA which helps in the transfer of this higher molecular weight DNA. If you allow the transfer to proceed for 20–24 hours, there will probably be no need to depurinate.

2. Transfer the gel to a tray containing denaturing solution (1.5 M NaOH/0.5 M NaCl).  
Place onto a shaking table top and shake gently for 20–30 minutes at RT. Ensure the gel is covered with liquid, as it may float.  
Replace the denaturing solution with fresh solution and shake gently for another 20–30 minutes.  
Wash the gel briefly in distilled water to remove traces of the denaturant.
3. Transfer the gel to a tray containing neutralising solution (1.5 M Tris-HCl, pH 7.0/0.5 M NaCl).  
Place onto a shaking tabletop and shake gently for 20–30 minutes at RT. Ensure the gel is covered with liquid, as it may float.  
Replace the neutralising solution with fresh solution and shake gently for another 20–30 minutes.  
The gel can remain shaking gently in neutralising solution for a while until you are ready to blot. When you are ready, wash the gel briefly in distilled water to remove traces of the neutralising solution.
4. Set up the blot as described below and following the diagram shown in the respective section of Sambrook et al. (1989).  
Fill a tray to about half way with 20 × SSC.  
Prepare a wick by cutting a sheet of Whatman 3 MM filter paper that is more than twice as long as the stage on which you will set up the blot. Wet this sheet with 20 × SSC.  
Place a stage in the tray of 20 × SSC and place wick over the top of the stage with the ends dipped into the 20 × SSC.  
Remove all air bubbles from the wick by rolling a glass pipette over the top.

For radioactively labelled probes, you can blot either onto neutral membranes (such as Hybond-N from Amersham) or positively charged membranes (such as Hybond N+ from Amersham). For DIG labelled probes, you must blot onto positively charged membranes. It is best to use the membrane supplied by Roche Diagnostics (formerly Boehringer Mannheim) since they have developed the DIG system and guarantee good results with their membrane.

5. Place the gel upside down on top of the wick.  
Remove all air bubbles.  
Trim off any parts of the gel that are not needed.

On loading a gel, the DNA sinks to the bottom of the well. Therefore, the DNA is actually closer to the underside of the gel. Hence you place it upside down for blotting since this means the DNA travels a shorter distance to get to the membrane.

6. Surround the gel with plastic wrap ensuring the wick is completely covered but the gel is not.

Placing plastic wrap around the gel prevents short-circuiting. If any of the paper towelling (see below) touches the wick, the buffer will travel to the towel directly without going through the gel and the DNA will not transfer efficiently.

7. Wearing gloves, cut a piece of nylon membrane to the size of the gel.

With a pencil, label one corner on the side of the membrane that will be in contact with the gel.

Fill a tray with  $2 \times$  SSC. Wet the membrane with this  $2 \times$  SSC by gently floating the membrane on the top until it has wet completely on the bottom and then gently submerge it.

Place the membrane on top of the gel, with the labelled side closest to the gel.

Remove all air bubbles by rolling a pipette over the top as before.

Discard the membrane and use a fresh one if it moves during this process.

8. Cut 2 sheets of 3MM filter paper to slightly bigger than the gel and membrane.

Wet these sheets with  $2 \times$  SSC.

Place the 2 sheets on top of the membrane.

Remove all air bubbles by rolling a pipette over the top as before.

9. Stack dry paper towelling over the top of the wet 3MM. The towelling should form an even, reasonably tidy stack to ensure good, even transfer.

Place a flat sheet of glass or plastic over the stack and on top of that place a weight of about 1 kg such as a 1 L flask filled with water.

10. Leave the transfer proceed for 20–24 hours.

11. The next day, remove the towelling and 3MM paper from the membrane.

Remove the membrane and immediately seal in plastic wrap to avoid it drying out.

Make sure the wrap is reasonably smooth on the DNA side.

Fix the DNA to the membrane by placing the membrane with the DNA side down (still in the plastic wrap) onto a UV transilluminator for 3 minutes. You can buy a box specifically for this purpose from BioRad ([www.bio-rad.com](http://www.bio-rad.com)).

Remove the membrane from the warp and wash in  $2 \times$  SSC to remove any traces of gel.

Immediately re-seal in a plastic bag to prevent it drying out.

Store at  $4^{\circ}\text{C}$  until ready for use.

12. Disassemble the remainder of the blotting apparatus.

The gel can be stained with ethidium bromide at this stage to check that DNA transfer has occurred.

If you do check the amount of DNA left on the gel, do not be surprised if you see high molecular weight DNA remaining in the gel. This is normal, the majority of the DNA will have transferred.

## **Southern hybridisation using DIG-labelled probes**

### ***Labelling probes with DIG***

DNA probes are labelled using either PCR or random priming (also known as oligo-labelling). Both methods require a DNA template from which complementary sequence is synthesised by a DNA polymerase such as *Taq* (in the case of PCR) or Klenow fragment (in the case of random priming).

Roche diagnostics (formerly Boehringer Mannheim) developed the DIG labelling system as an alternative to radioactive labelling. They have published a technical manual for the labelling, use and detection of DIG labelled probes (see The DIG System User's Guide for Filter Hybridisation; <http://biochem.roche.com>). The labelling reaction incorporates digoxigenin-11-dUTP (DIG-11-dUTP). The molar ratio of the DIG-11-dUTP:dTTP determines in part the specific activity of the final product. Different products are available with 1:2 and 1:20 ratios. For probes to be used for Southern analysis, a higher level of DIG-dUTP incorporated per amount of template DNA (i.e. a higher specific activity) is generally required, especially for the detection of single copy genes within complex genomes. To make such probes, the lower ratio (i.e. 1:2) works better.

### ***Hybridisation and washing***

The hybridisation temperature used depends largely on the probe and the DNA you are probing. If using a probe with 100% homology to the target sequence, such as in the case of transgene detection, 50°C is an appropriate temperature.

#### **1. Put on gloves and WASH TO REMOVE ANY TRACES OF TALCUM POWDER.**

Talcum or other powder can cause spotting background when you come to detect the hybridisation signal.

Pre-warm DIG Easy Hyb solution to 50°C.

Pre-warm hybridisation oven (for bottles) or shaking water bath (for plastic bags) to 50°C.

#### **2. Place membrane into a plastic bag or hybridisation bottle.**

Hybridisation bottles are preferred since these are less messy than using plastic bags and good rotation of the membrane with the hybridisation solution can be assured. The use of bottles requires that you have an appropriate hybridisation oven that can rotate the bottles. If you are using plastic bags, ensure they are submerged into the water of the shaking water bath, otherwise the hybridisation will not be even. You can do this by using a small lead weight to hold them down at each corner.

You can have more than one membrane per bottle/plastic bag. Use nylon gauze between each membrane to ensure they do not stick to each other and the hybridisation solution can reach each one.

#### **3. Place 10 mL of pre-warmed DIG Easy Hyb solution in with the membrane, close bottle ensuring no leakage of solution through the seal. Seal bag if using plastic bags, ensuring the solution does not leak out.**

Place into hybridisation oven or shaking water bath at 50°C.

Prehybridise by rotating or shaking the membranes in the solution for at least 30–60 minutes at 50°C.

4. If using fresh, previously unused probe: in a sterile Eppendorf tube, add 10–20 µL of DIG-labelled probe directly to 1 mL of DIG Easy Hyb solution.

Denature for 10 minutes by placing tube in a boiling water bath. Ensure the lid of the tube does not pop open during boiling as you may lose or dilute the probe.

Quench on ice immediately.

Discard DIG Easy Hyb solution, used for prehybridisation and add 5–10 mL fresh pre-warmed DIG Easy Hyb.

Add denatured, quenched probe to this solution in the bottle/plastic bag by pipetting.

Do not pour the solution in as you may pour it onto the membrane. If this occurs, you are likely to get a big blob of background where it hits the membrane.

DIG-labelled probes can be re-used several times. When using a DIG-Labelled probe for the first time, its activity needs to be determined. That is, you need to determine how well the probe has been labelled with the DIG label. Refer to the DIG user's manual for details.

If using a previously used probe: replenish the used DIG-labelled probe by adding 25% unused probe.

Boil for 10 minutes by placing the tube in a boiling water bath. Ensure the lid of the tube does not pop open during boiling as you may lose or dilute the probe.

Quench on ice.

Remove the prehybridisation solution from the bottle/plastic bag.

Pour in the boiled hybridisation solution.

## 5. Hybridise overnight at 50°C by rotating bottle/shaking plastic bag.

Following hybridisation, pour off the probe into a fresh tube and store at –20°C until needed again.

If using bottles, you can carry out the following washing steps with the membrane still in the bottle. If using plastic bags, remove the membrane from the bag and wash in trays. The trays will need to be as small as possible and with lids so that the water of the shaking water bath does not accidentally get into the trays and damage the membranes.

## 6. Wash membranes at RT.

These washes are carried out to remove excess probe from the membrane and to remove any non-specific hybridisation of the probe to sequences other than the target sequence. The first wash is at low stringency (i.e. high salt, low temperature) to remove the excess probe. The second wash is at high stringency (i.e. low salt, high temperature) to eliminate any non-specific binding. Depending on the homology of your probe for the target sequence and the length of the probe, you can adjust these washing conditions. If you are trying to hybridise a probe with low homology to the target sequence, you may wish to stop at the low stringency wash. Remember that with DIG-labelled probes, unlike with radioactively labelled probes, you cannot detect after the first round of washing and then do more washes to increase the stringency. Once you have detected the hybridisation, the only thing you can do is to strip the probe off and rehybridise, washing at a different stringency the next time.

**Bottles:** Set a hybridisation oven to 68°C for later high stringency washes.

Pour in ~100 mL of 2 × SSC/0.1% SDS.

Rotate the bottles quickly at RT for 5 minutes.

Pour off the solution and add fresh solution.

Repeat wash twice at RT for 5 minutes each.

**Plastic bags/trays:** Set a water bath to 68°C for later high stringency washes.

With membranes in a washing tray, pour in 2 × SSC/0.1% SDS to ensure membrane is well covered.

Cover tray with lid and place on shaking tabletop for 5 minutes at RT.

Pour off the solution and add fresh solution.

Repeat wash twice at RT for 5 minutes each.

7. Replace 2 × SSC/0.1% SDS with 0.2 × SSC/0.1% SDS.

Carry out 2 × 20 minutes washes as described above, this time at 68°C.

8. Cool oven/water bath to RT.

Rinse membrane in 0.2 × SSC/0.1% SDS at RT to cool blot and bottle.

The blot needs to be cooled at this stage since if it is hot, the blocking solution to be used in a later step will curdle and rendered ineffective.

### ***Detection of the hybridisation signal***

If using bottles, you can carry out the detection steps with the membrane still in the bottle. If using plastic bags, continue to use trays.

1. Bring the 10 × blocking solution to room temperature (this is usually stored at -20°C)  
Equilibrate the wet membrane with 10 mL of 1 × DIG wash buffer.  
Wash 1–2 minutes at RT.
2. Prepare 2 × blocking solution by diluting the 10 × stock 5-fold in 1 × maleic acid buffer.  
Discard the wash buffer and replace with 20 mL of 2 × blocking solution brought to RT.  
Agitate for 60 minutes at RT.
3. Centrifuge anti-DIG alkaline phosphatase-conjugated antibody in a microfuge for 5 minutes at RT.

This ensures any precipitate or particulate matter is at the bottom of the tube and not accidentally pipetted onto the membrane where it can contribute to background noise.

Pipette 1 µL of the antibody conjugate directly into the blocking solution already in the bottle/tray (this gives a 1:20 000 working dilution).

Rotate/shake at RT for 30 minutes.

Do not add directly onto the membrane as the concentrated antibody will cause uneven detection and high background in that spot. There are different chemiluminescent substrates available for the alkaline phosphate conjugated antibody. CSPD does not provide as sensitive a detection (ca. 25 × less) as CDP-Star. For CSPD, dilute the antibody 1:10 000; for CDP-star dilute the antibody 1:20 000.

**4. Discard the antibody solution from the bottle/tray.**

Wash the membrane in 100 mL of 1 × DIG wash buffer, twice for 15 minutes each at RT. Remove substrate from storage and bring to RT, ensure there is no precipitate in the solution.

**5. Wash membrane for 2–5 minutes in 10 mL 1 × DIG detection buffer to equilibrate the membrane to the new buffer.**

**6. Mix 100 µL of CSPD or CDP-star with 10 mL of 1 × detection buffer, i.e., dilute 1:100.  
ENSURE POWDER IS REMOVED FROM GLOVES BY WASHING**

**7. Stretch some plastic wrap over the bench, taping each of the corners down to obtain a smooth surface.**

Remove membrane from bottle/tray and place, DNA side up onto the plastic wrap.

Cover each membrane with 5 mL of diluted substrate prepared in step 6.

Leave 5 minutes on the bench at RT, ensuring the filter is evenly wet and does not lift during this period.

**8. Drip membranes dry and blot back dry onto 3MM paper briefly.**

Place damp membranes between 2 sheets of overhead transparency ensuring no air bubbles are present. Do not rub membranes as this may smudge the signals.

For CSPD substrate, place at 37°C for 10 minutes. This is not necessary for CDP-star substrate.

Expose to x-ray film in a film cassette without intensifying screens at RT for 1–2 hours initially.

Develop film and re-expose to film for shorter or longer periods, if necessary.

Use write-on transparencies you use with an overhead projector. Ensure that the transparencies you use have the same texture on each side. Do not use those with one rough side (photocopier film), as they stick fast together and mask the signal and you will not detect anything.

## **Southern hybridisation using radioactively-labelled probes**

### ***Labelling probes with radioactivity***

As for DIG-labelled probes, radiolabelled DNA probes are made using either PCR or random priming (also known as oligo-labelling). The most commonly used label is [ $\alpha^{32}\text{P}$ ]-dCTP although [ $\alpha^{33}\text{P}$ ]-dCTP is also used.

Most molecular biology companies such as Pharmacia, Roche Diagnostics etc. supply kits for the labelling of DNA probes. We recommend the use of these since the preparation of the buffers etc required for oligolabelling can be tedious. The use of kits has simplified and sped up considerably the process of preparing radiolabelled probes.

### ***Hybridisation and washing***

As for DIG-labelled probes, membranes can be hybridised in either bottles or plastic bags. If at all possible, use hybridisation bottles, as they are much easier to handle than plastic bags. The introductory notes for using DIG-labelled probes regarding hybridisation temperature also apply to the use of radioactively labelled probes; however, the hybridisation method described here uses an aqueous hybridisation solution unlike the DIG Easy Hyb. Hence, the temperature of hybridisation will be higher to achieve the desired stringency.

1. Pre-warm hybridisation oven (for bottles) or shaking water bath (for plastic bags) to 65°C.
2. Place membrane into a plastic bag or hybridisation bottle.
3. Place 10 mL of hybridisation solution in with the membrane, close bottle ensuring no leakage of solution through the seal. Seal bag if using plastic bags, ensuring the solution does not leak out.

Place into hybridisation oven or shaking water bath at 65°C.

Prehybridise by rotating or shaking the membranes in the prehybridisation solution for a minimum of 60 minutes (up to overnight is fine, 4 hours is probably better than 1 hour) at 65°C.

4. Have freshly labelled probe in an Eppendorf tube.

Add 100 µL of 10 mg/mL sheared herring or salmon sperm DNA.

Denature for 10 minutes by placing tube in a boiling water bath. Ensure the lid of the tube does not pop open during boiling.

Quench on ice.

Either add directly to the prehybridisation solution in the bottle/plastic bag by pipetting or replace the prehybridisation solution with a fresh 10 mL to which the denatured herring sperm DNA has been added.

Herring sperm or salmon sperm DNA is added to reduce the background. This DNA binds non-specifically to the membrane, preventing the radioactively-labelled probe from doing so.

Do not pour the solution in as you may pour it onto the membrane. If this occurs, you are likely to get a big blob of background where it hits the membrane.

5. Hybridise overnight at 65°C by rotating bottle/shaking plastic bag.

Following hybridisation, discard the radioactive hybridisation solution appropriately.

Radioactively-labelled probes can be reused, depending on the isotope used and the specific activity of the probe. If it is a very 'hot' probe made with fresh isotope, then it can probably be reused within the next 2 weeks.

6. Washing is carried out as described for DIG-labelled probes with the following modifications:

Wash 2 × 20–30 minutes at 65°C with 2 × SSC/0.5% SDS.

Wash 2 × 20–30 minutes at 65°C with 0.2 × SSC /0.5% SDS.

### ***Detection of the hybridisation signal***

After washing, remove membrane from bottle or tray and seal in plastic (removing air bubbles) before it dries out.

Using a Geiger counter, monitor the level and location of radioactivity. Compare with a corner of the membrane, which should be 'cold'.

Depending on the level of radioactivity, expose to x-ray film in cassettes using intensifying screens up to overnight at -80°C

Develop film and re-expose to film, if necessary.

### **3.3 Transgene expression: mRNA**

Analysis of transgenic plants at the RNA level allows the researcher to determine the expression of the gene transferred into the plant species of interest. Genes are transcribed into messenger RNA (mRNA) which is then, in turn, translated into protein. Thus, a measure of the mRNA level from a specific gene gives a measure of the transcriptional activity of that gene i.e., when the mRNA is present, the gene must be transcriptionally active.

The presence of a specific mRNA molecule can be detected by many different methods. Here we briefly describe the use of northern hybridisation using DIG-labelled and radioactively-labelled probes as well as reverse transcription -PCR (RT-PCR) for measuring the cellular steady state levels of mRNAs. The rate of transcription of a specific mRNA in the cells' nuclei can also be measured by an assay called 'nuclear run on'. We do not describe this here but if you wish to find out more information, please refer to Current Protocols in Molecular Biology (Ausubel et al. 1997).

Northern hybridisation is similar to Southern hybridisation. Either total RNA or poly(A)<sup>+</sup> RNA (mRNA is usually polyadenylated to direct it into the cytoplasm) is extracted and separated according to size via denaturing agarose gel electrophoresis. The RNA is then transferred to a nylon membrane by blotting and hybridised with a mRNA-specific probe which has been labelled with either a radioisotope or with a non-radioactive label such as digoxigenin (DIG). This hybridisation is then detected by the appropriate method for the label used.

Detection of specific mRNA molecules from a total RNA population relies on the mRNA being reasonably abundant. If the mRNA is not abundant, the sensitivity of this procedure may not be sufficient. Thus, it may be necessary to purify the mRNA by isolating polyA<sup>+</sup> RNA.

The presence of a transgene transcript can also be detected by RT-PCR. This is a similar procedure as described for genomic DNA analysis except that the RNA is first used as a template for synthesising cDNA, which is then used as the template for the PCR. Please refer to Dietzgen (2000) for a detailed description of RT-PCR.

### **Preparing an RNase-Free Environment**

RNA is very susceptible to degradation by ribonucleases (RNases). RNases are very stable enzymes that generally require no cofactors to function. Thus, even very small quantities of RNase contamination can cause serious degradation of the RNA. RNases can come into contact with RNA when cells are lysed during extraction procedures as well as from the outside from the researcher's hands, solutions and equipment. Thus, it is important to try to maintain an RNase-free environment when preparing and working with RNA. This is almost impossible; however, there are things that can be done to reduce the risk of contamination. The most important aspect of working with RNA is being aware of the where the risks of contamination are and trying to avoid them. There is a good description of RNase contamination and how to avoid it in Sambrook et al. (1989). It is not difficult to work with RNA if you have planned and prepared well and if you are aware of potential contamination as you work.

As much as possible, use sterile unused disposable plasticware. Such plasticware is free of RNases and can be used without pre-treatment. All other plasticware and glassware must be treated prior to use to remove contaminating RNases. It is especially important to remember that as you work in the laboratory, you will come into contact with many surfaces that are potentially contaminated. Hands are also a major source of RNases, thus wear gloves and be aware of what you have touched, changing your gloves regularly. Sterilise your work area.

Use fresh, sterile solutions that are dedicated to RNA work; using the same solutions for RNA and DNA work increases the risk of RNase contamination. You can also reduce the risk of contamination by using only sterile pipette tips, graduated pipettes or sterile measuring cylinders etc for dispensing solutions for RNA work. While this is not necessary, if you are having problems with RNase contamination, you may wish to reserve chemicals, glass and plasticware and equipment for RNA work.

The minimal pre-treatment is to sterilise by autoclaving, although this does not remove all RNases. Methods to prepare RNase-free glassware (baking 240°C) and plasticware and solutions (using the chemical 'diethylpyrocarbonate', DEPC) are described in Sambrook et al. (1989). Be careful when handling DEPC as it is a suspected carcinogen. DEPC cannot be used for solutions containing Tris because it reacts with amines. For solutions containing Tris, make up solutions with DEPC-treated water in an RNase-free bottle and add to RNase-free solutions. If possible, reserve a bottle of Tris crystals for RNA work.

## Northern Hybridisation Analysis

RNA can be purified from the rest of a cell's components using one of several procedures. As for extraction of genomic DNA, the methods for RNA extraction involve disrupting the plant cells to release their contents followed by the denaturation and removal of proteins, carbohydrates and cell debris, usually with phenol/chloroform. During cell lysis, RNases must be denatured to render them inactive. In the method described below, phenol and detergent are used to achieve this. An alternative is to use guanidinium isothiocyanate. The higher molecular weight nucleic acids (chromosomal DNA) are precipitated leaving the lower molecular weight nucleic acids (including RNA) behind. The RNA is then precipitated, or alternatively it is pelleted through a caesium chloride cushion. The RNA purification methods described below have been used for the purification of total RNA from peanut.

Northern hybridisation was developed after Southern hybridisation. Its name refers to the transfer of RNA, rather than DNA, to a solid support, such as a membrane, and hybridisation of that RNA with a DNA or RNA probe. The transfer is done as it is done for Southern blots. The major difference is that rather than denaturing the nucleic acids after gel electrophoresis, RNA is denatured prior to loading the gel and during gel electrophoresis. Northern analysis gives a measure of the steady state level of a specific mRNA and its size. It does not give a measure of the transcription rate of the mRNA nor the structure of this molecule.

### Total RNA extraction

1. Remove ~1 g of plant tissue and wrap in labelled aluminium foil (record weight). Freeze in liquid nitrogen immediately. The tissue can be extracted straight away or stored frozen at -80°C until required.

2. Place tissue in mortar and add liquid nitrogen.
3. Working in a fume hood, grind tissue in liquid nitrogen to a fine powder ensuring no large bits of tissue remain.  
Add 2 mL NTES per 1–2 grams of tissue and 1.5 volumes (1.5 mL/g) of phenol/chloroform/isoamyl alcohol.  
Grind tissue until it has thawed and become totally liquid.  
Pour into a polypropylene tube such as a Falcon tube (15 or 50 mL) that can be capped well and will be less than half full with the extract.  
Vortex vigorously for 5 minutes.
4. Separate the phases by centrifugation for 15 minutes at 4°C at top speed in a benchtop centrifuge. A swing out rotor is best; 12 000 × g is the ideal speed. If the centrifuge cannot spin at this speed, increase the time.  
In a fume hood, remove upper aqueous phase to a sterile Oakridge tube (ensure no phenol is transferred).
5. Add 0.1 volume 2 M sodium acetate pH 5.8 and 2 volumes of absolute ethanol.  
Leave overnight at –20°C.
6. Spin at 9000 r/min in a Beckman JA20 rotor or equivalent for 20–30 minutes at 4°C.  
Pour off the ethanol carefully, ensuring the pellet remains in the tube.  
Drain by standing tube upside down on tissue, ensuring the pellet remains in the tube.  
Wash the pellet with 70% cold (–20°C) ethanol.  
Centrifuge for 5 minutes at 12 000 × g at 4°C.  
Pour off 70% ethanol carefully, ensuring the pellet remains in the tube.  
Drain by standing tube upside down on tissue, ensuring the pellet remains in the tube.  
Air-dry briefly.
7. Dissolve pellet in 1 mL of sterile distilled water per 1–2 g of plant tissue.  
Add 1 volume of 4 M LiCl (or 0.25 volume of 10 M LiCl).  
Stand for 3 hours on ice, or overnight at 4°C.  
Centrifuge at 12 000 × g for 10 minutes at 4°C.
8. Pour off supernatant carefully, ensuring the pellet remains in the tube.  
Dissolve pellet in sterile distilled water (0.5 mL/g tissue).  
Add 0.1 volume of 2 M sodium acetate pH 5.8 plus 2 volumes of absolute ethanol.  
Leave at –20°C for more than 2 hours.  
Centrifuge at 12 000 × g for 10 minutes at 4°C.  
Wash pellet with 70% ethanol.  
Centrifuge for 5 minutes at 12 000 × g at 4°C.  
Pour off 70% ethanol carefully, ensuring the pellet remains in the tube.  
Air-dry briefly.
9. Dissolve pellet in sterile distilled water (200 µL/g tissue).  
Determine the concentration from the  $A_{260}$  measurement. An OD of 1 is equivalent to 40 µg/mL of single stranded RNA.  
Once the concentration of the RNA is known, calculate the total amount of RNA present within the tube, i.e. the yield.

Before measuring the  $A_{260}$ , ensure the RNA is fully resuspended. The purity of the RNA can be determined by calculating the  $A_{260}/A_{280}$  ratio. If it is 1.8–2, then the RNA is pure, if it is less then it has protein and/or phenol contamination and the concentration measurement will not be accurate.

10. Store RNA precipitated under ethanol, i.e. add 0.1 volume of 2 M sodium acetate pH 5.8 and 2 volumes of absolute ethanol. Store at either  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$ .

11. When you wish to remove some RNA, place onto ice when removing from freezer. Vortex thoroughly.

Remove the volume equivalent to the amount of RNA you wish to analyse and transfer to a fresh, sterile Eppendorf tube.

Place the stock RNA back in the freezer.

Centrifuge the RNA you have removed at  $12\,000 \times g$  at  $4^{\circ}\text{C}$  for 20–30 minutes.

Wash pellet with 70% ethanol.

Centrifuge for 5 minutes at  $12\,000 \times g$  at  $4^{\circ}\text{C}$ .

Pour off 70% ethanol carefully, ensuring the pellet remains in the tube.

Air-dry briefly.

Resuspend in the appropriate volume of sterile distilled water to give the RNA concentration you require.

## Poly(A)<sup>+</sup> RNA extraction

mRNA represents only about 1% of the total RNA within a cell. If you find that you cannot detect a specific mRNA from within a total RNA population, it maybe that this mRNA is not very abundant and the sensitivity of the assay is not high enough. The sensitivity can be increased using the mRNA population only. With rare exceptions, mRNA molecules have a string of adenine residues at their 3' end known as a polyA tail. Affinity chromatography based on the hybridisation of adenine and thymidine is used to isolate mRNA, thus, the polyA tail is used as kind of hook for separating mRNA from the other RNA forms. A total RNA population is combined with oligo(dT) molecules bound to a solid matrix such as cellulose. The mRNA molecules then become physically separated from the other RNA forms as their polyA tails hybridise to the oligo(dT). Obviously, for the purification of poly(A)<sup>+</sup> RNA, it is critical that the starting RNA be intact and undegraded.

A method describing the isolation of mRNA can be found in Sambrook et al (1989). However, many molecular biology companies sell products specific for the purification of mRNA. These products have simplified and sped up this procedure greatly.

## Agarose gel electrophoresis of RNA

Some researchers recommend having gel electrophoresis equipment set aside for RNA work only to reduce the risk of contamination with RNases. If you do not have the resources for this, it is possible to use equipment for common purposes for RNA work. Ensure the gel tank, tray and comb are cleaned with detergent and rinsed thoroughly in DEPC-treated water. Wipe with ethanol and air dry. Some recommend then soaking the equipment in 3%  $\text{H}_2\text{O}_2$  for 10 minutes at RT and rinsing thoroughly with DEPC-treated water (Sambrook et al. 1989).

Even though RNA is a single stranded molecule, it can fold into secondary structures via intramolecular base pairing forming double stranded regions. To probe RNA sequences effectively, this secondary structure must be denatured. This is done prior to loading RNA onto an agarose gel. To prevent the RNA from refolding as it migrates through the agarose, a denaturing gel containing formaldehyde can be used.

**1. Prepare deionised formamide and formaldehyde.**

In a fume hood, add 1 g of Mixed bed resin to 100 mL of formamide/formaldehyde.

Stir gently for 30 minutes and filter through Whatman #1 paper into sterile containers such as 50 mL Falcon tubes.

Formamide and formaldehyde are hazardous chemicals and as such should be treated with caution by wearing a lab coat, gloves and working with them in a fume hood. They need to be deionised since ions present within these solvents can negatively affect the way the RNA migrates through the agarose gel. Greater than 100 mL can be deionised and stored at -20°C for future use.

**2. Up to 5 µg of total RNA will be loaded onto the gel, thus, remove the volume equivalent to 5 µg from your RNA stock and prepare as described above, resuspending finally in 3.5 µL of sterile distilled water.**

Generally, 5 µg of total RNA has been sufficient for several legume species, including peanut, for the detection of specific mRNAs within a total RNA population. If you wish, you can load >5 µg by scaling up the preparation of the sample as described below. If you do this, ensure the gel well can hold the increased volume.

**3. Prepare the gel tray before making up the gel mixture. See Chapter 3.2 for notes on this.  
Prepare a 65°C water bath.**

**4. Using a sterile bottle/flask to make up a 100 mL gel, melt 1.4 g of high quality agarose in 10 mL of 10 × MOPS buffer and 85 mL of sterile, RNase-free distilled water.**

Melt in a microwave oven or over a Bunsen burner without over boiling.

Check the volume by using a sterile plastic measuring cylinder.

Make up to 95 mL with sterile, RNase-free distilled water.

Pour back into bottle/flask.

In a fume hood, add 5 mL of deionised formaldehyde and mix well.

In one action, pour the agarose mixture into the prepared gel tray that is waiting in the fume hood.

Remove air bubbles from the gel with sterile pipette tip or Pasteur pipette.

Leave to set at RT.

**Caution:** Ensure you are wearing safety glasses, gloves and a lab coat when handling formaldehyde-containing solutions. Do not put your face over the gel after it is poured to avoid inhaling formaldehyde fumes.

**5. While the gel is setting, prepare 1 × MOPS buffer from 10 × stock. Depending on the gel rig you are using, you may need up to ~1L of this.**

Also prepare the sample denaturing solution. For each sample, mix together in a sterile Eppendorf tube:

10 µL deionised formamide.

3.5 µL deionised formaldehyde.

2 µL 10 × MOPS buffer.

1 µL 10 mg/mL ethidium bromide.

Mix thoroughly and leave on ice until required.

6. Place the set gel into the gel rig, ensuring any tape and the comb have been removed.  
Pour 1 × MOPS buffer into the gel rig so that it is level with the top of the gel but does not submerge the gel.
  7. Mix together 3.5 µL (= ~5 µg of total RNA) and 16.5 µL of the sample denaturing solution.  
Place in the 65°C water bath for 5 minutes.  
Quench on ice.  
Add 5 µL of RNA loading dye and load immediately onto gel.  
Other samples to load would be positive and negative controls as well as RNA size markers.  
Assemble the gel rig and hook it up to a power pack, remembering that RNA being negatively charged at neutral pH will run towards the positive electrode.  
Start the gel running by setting the desired voltage.  
Slowly electrophorese the RNA about 1 cm into the gel.  
Turn off the current and pour in more 1 × MOPS buffer to submerge the gel ~1 cm.  
Turn the current back on and run until the bromophenol blue has reached ~1 cm from the bottom of the gel.
8. If you are using a horizontal gel rig, remove the gel and check how far the RNA has run and its integrity.  
If the RNA has run far enough, take a photograph of the gel as a record.  
If you have run unlabelled markers, mark the position of each marker and/or the rRNA bands which should be clearly visible. This can be done using Indian ink or cutting the gel as described in chapter 3.2. Once the rRNA has been transferred to the membrane, they should be clearly visible. You may wish to wait and mark their position in pencil on the membrane after blotting.  
You should also include a negative control such as RNA prepared from an untransformed plant, and a positive control such as RNA from a plant you know is expressing the RNA or a synthetic RNA prepared from the cloned gene.

## Northern blotting

1. Transfer the gel from the gel rig to a tray.  
Wash gently in sterile, RNase-free distilled water for 15 minutes.  
Replace the water with 20 × SSC and wash for 20 minutes at RT.  
Repeat the 20 × SSC wash.
2. Set up the blotting rig as described for Southern blotting (see page 53).  
The next day, disassemble the blotting apparatus as described.  
Remove the membrane and immediately note the positions of the rRNA bands (which should be visible) with a pencil on the side of the membrane. Then cover in plastic wrap to avoid it drying out.  
Fix the RNA to the membrane as described for Southern membranes.  
Remove the membrane from the wrap and wash in 2 × SSC to remove any traces of agarose.  
Immediately seal in a plastic bag to prevent it drying out.  
Store at 4°C until ready for use.
3. Look at the gel after transfer on a UV transilluminator to confirm that most of the RNA was transferred.

## **Northern Hybridisation**

### ***Labelling probes***

DNA probes are labelled either with DIG or radioactive isotopes as described for Southern hybridisation. RNA probes (riboprobes) are often used for probing northern blots as these probes provide greater sensitivity than DNA probes and allow strand specific detection of mRNA. To make an RNA probe, you must have your sequence of interest cloned into a plasmid that carries a promoter for a DNA-dependent RNA polymerase such as T7, T3 or SP6. Also, the sequence must be cloned in such a way so that transcription from the promoter through the sequence of interest will give rise to anti-sense (complementary) synthetic RNA that can then hybridise to the sense mRNA molecule within the RNA found on the membrane. If you transcribe a sense RNA probe, it will not hybridise to the mRNA but rather to any complementary antisense RNAs that may be present. For more information about the desired features of DNA templates for preparing riboprobes, please refer to the technical information provided by the various companies that supply the relevant kits.

While DIG-labelled probes have been used for the detection of mRNAs on northerns, the sensitivity may not be as high as you need. Thus, we recommend the use of radioactively-labelled probes for northern hybridisation analysis. In doing so, we also urge you to ensure you are adequately prepared for the use of radioisotopes.

### ***Hybridisation and washing***

Hybridisation is carried out essentially as described for Southern hybridisation. The difference is the hybridisation mixture and/or temperature of hybridisation. Generally, hybridisation solutions containing formamide are used in northern hybridisation analysis. We recommend the use of a formamide containing hybridisation solution and hybridisation at 42°C as a starting point. Always include RNA prepared from an untransformed plant as a negative control on your northerns, this will tell you if you have specific hybridisation to the transgene or not.

Carry out hybridisation and washing according to the instructions outlined for Southern hybridisation analysis, substituting 42°C for the prehybridisation and hybridisation temperatures and substituting the following mixture for the Southern hybridisation solution:

- 50% formamide.
- 5 × SSC.
- 2 × Denhardt's solution.
- 0.1% SDS.
- 100 µg sheared herring sperm DNA/mL.

### ***Detection of the hybridisation signal***

Detection of the hybridisation signal is as described for Southern hybridisation (Chapter 3.2).

## **3.4 Transgene expression: protein**

Analysis of the transgene product provides information about the activity of the transgene within a transformed plant. If the transgene has been engineered to give rise to a protein, a measure of the specific transgene product gives a measure of gene activity as well as the translatability of the transgene mRNA. Synthesis of the transgene product provides further proof of the transcriptional activity of the transgene.

The presence of a specific protein can be detected by many different methods, most of which rely on the use of an antibody specific for the particular protein of interest. We have used plate-trapped antigen enzyme-linked immunosorbent assay (ELISA) as well as western or immunoblotting to measure the steady state levels of transgenic protein.

Plate-trapped antigen ELISA (PTA-ELISA) involves coating polystyrene wells with crude plant extracts and washing away any excess material before binding of the protein-specific antibody. This antibody is then detected by a secondary antibody, which has been covalently linked to an enzyme. The substrate for this enzyme is added and hydrolysed to yield a coloured soluble product which is detected spectrophotometrically. The method is semi-quantitative, i.e. the intensity of colour is an indication of the amount of specific protein present within the crude preparation.

Immunoblotting or western blotting is similar to southern and northern blotting in that target molecules (here: proteins) from a crude mixture are separated according to size by denaturing gel electrophoresis and then transferred to a membrane. The specific protein is detected using a specific antibody, like in ELISA. Again, a secondary antibody is applied which is conjugated to an enzyme. The enzyme's substrate is added and the resulting insoluble product either emits light energy or is coloured.

## **Plate-Trapped Antigen (PTA) — ELISA**

1. Remove 3 samples of ~5 mm<sup>2</sup> per leaf (cork borer No.4) and place into a labelled sterile Eppendorf tube.

Place immediately on ice. Use immediately or store at -20°C until required.

2. Add 400 µL coating buffer and grind in the tube using a small pestle. Place samples on ice while grinding the remaining samples.

Centrifuge for 2 minutes in a microfuge and return to ice.

Transfer 100 µL of each sample to a well of an ELISA plate in duplicate.

Place wet paper towels into a plastic box, lying them flat.

Place ELISA plate into box on top of wet paper towels and place lid on box.

Incubate for ~ 16 hrs at 4°C overnight.

In this assay, we use flat bottom polystyrene ELISA plates with a 96-well format. Please note that the plastic used to manufacture 96-well plates and the shape of the wells can vary. The wet paper towels are used to prevent the liquid from evaporating.

3. Place some PBS-Tween into a wash bottle.

Empty contents of plate by inverting the plate and shaking into a sink.

Wash plate with PBS-Tween by squirting solution vigorously into each well. All the green colour should disappear after this first wash. Ensure wells are left filled with PBS-Tween.

Leave 3 minutes at RT. Empty contents by flicking plate. Repeat PBS-Tween washes/ incubations twice.

Finally empty wells by flicking the plate and with a vigorous action, slam the plate down 5 times onto some cushioned paper towels without breaking the plate.

It is important that as much liquid is removed from each well as possible between each wash. This reduces the amount of carry over between treatments.

4. Remove antibody (usually prepared from a rabbit) from freezer and dilute appropriately in sample buffer. For a good antiserum, we use a 1:1000–1:10 000 dilution.  
Add 100 µL of diluted antibody to each well.  
Incubate for 2 hours at RT in the boxes with wet paper towels.
5. Wash with PBS-Tween as described above.
6. Dilute commercial secondary antibody (goat anti-rabbit IgG-alkaline phosphatase) in conjugate buffer as recommended by the manufacturer.  
Add 100 µL to each well.  
Incubate in the boxes for 3 hours at RT.

This secondary antibody is generally purchased from a company such as Sigma. We have used a goat anti-rabbit IgG since the primary antibody was raised in a rabbit. It does not matter what the source of the secondary antibody is as long as it is specific for the species in which you raised the primary antibody. The dilution at which to use this antibody will be recommended by the manufacturer. It is generally in the order of 1:10 000–1:30 000.

7. Wash with PBS-Tween as described above.
8. Prepare a fresh 1 mg/mL solution of p-nitrophenyl phosphate in substrate buffer.  
Add 100 µL to each well.  
Incubate for 1 hour at RT.

This incubation time can be from 15 minutes to 2 hours, depending on how fast colour develops — you need to watch and decide empirically. Whatever length of time you choose, you should use the same length of time for all your experiments so you can compare your data. Depending on what is available in your laboratory, other enzymes such as peroxidase or penicillinase and their respective substrates and buffers can be used.

9. Stop reaction by adding 50 µL of 1 M NaOH per well.
10. Measure the A<sub>410/405nm</sub> values using an ELISA plate reader.

For this particular substrate, you will notice the colour turning yellow where you have binding of the antibody to the protein of interest. Hence, the absorbance is read at 410 or 405 nm. When you carry out a PTA-ELISA experiment, ensure you have included positive and negative controls. A negative control would be a leaf extract from an untransformed plant and buffer only. This will tell you the non-specific background binding of the antibody to plant proteins or the plastic. A positive control would contain the specific protein that the antibody detects, i.e. in the case of an expressed viral coat protein, an extract from a virus-infected plant could be used.

## Western Blotting

### SDS-polyacrylamide gel electrophoresis

This procedure for electrophoresis of proteins is based on Laemmli (1970).

We recommend using a Mini Protean II System available from BioRad for the electrophoresis of samples and the subsequent electroblotting of these to nitrocellulose membrane.

1. If using a Mini-Protean II System, assemble the gel plates as described by the manufacturer. If using home made plates, put together the gel plates by taping and clamping so as to avoid leakage.

2. Prepare a lower (separating) gel as follows:

In a 50 mL beaker, mix together distilled water, lower Tris buffer and acrylamide/bisacrylamide (29:1) mixture according to the proportions outlined below for the percentage gel you wish to prepare.

When you are ready to pour the gel, add the freshly prepared 10% ammonium persulphate stock and TEMED, swirl to mix while avoiding the formation of air bubbles.

	7.5%	8%	10%	12%	15%
Distilled water	6.63 mL	6.4 mL	5.53 mL	4.63 mL	3.3 mL
Lower Tris	3.33 mL				
Acryl/bis	3.33 mL	3.56 mL	4.43 mL	5.33 mL	6.67 mL
10% amm. persulfate	40 µL				
TEMED	7 µL				

The recipes given above are for the Mini-Protean II System. This size gel is generally suitable for the analysis of transgenic plants; however, if you wish to obtain greater resolution, use a larger gel system and scale up the above recipes.

Smaller proteins will migrate through the gel more quickly than larger ones during electrophoresis. For analysis of proteins of 25–50 kDa, use a 10% gel; for smaller proteins up to 10 kDa, 12% is recommended.

3. Add the mixture by pouring it carefully between the plates, using a wide-bore pipette. Try to avoid trapping air bubbles, move the plates about to encourage any air bubbles to the top of the gel mixture. Ensure the separating gel mix does not go above ~ 1.5–2 cm (a finger wide) from the top.  
Overlay the mixture by carefully pipetting some water-saturated butanol over the top with a Pasteur pipette.  
Allow the mixture to polymerise for 1 hour at RT.

The butanol is to provide an anaerobic environment for the gel to set and an even surface on which to pour the stacking gel. To saturate butanol with water, add ~10% (v/v) distilled water, shake vigorously and wait until the phases have separated. The butanol will be on the top.

4. Prepare a 5% upper (stacking) gel as follows: In a 20 mL beaker mix 1.93 mL distilled water, 0.85 mL Upper Tris buffer, and 0.58 mL acrylamide/bisacrylamide (29:1) solution.
5. Pour off the butanol from the polymerised separating gel and, using a wash bottle, carefully rinse the surface of the gel with distilled water and remove any excess liquid with an absorbent tissue.

When you are ready to pour the stacking gel, add 20 µL 10% ammonium persulphate and TEMED, swirl to mix avoiding the formation of air bubbles and pour immediately (the gel should set within 10–15 minutes).

Carefully and quickly pour the stacking gel mixture to the top of the separating gel until it reaches ~2 mm from the top of the plate. Try to avoid trapping air bubbles.

Carefully insert the comb into the stacking gel mixture, trying not to trap air bubbles at the bottom of the comb's teeth.

Allow to polymerise for 30 minutes.

6. Once the gel has polymerised, assemble gel rig according to the manufacturer's instructions. If you are using a home made rig, assemble so that no buffer will leak from the upper reservoir into the lower reservoir. You may need to use Vaseline to seal the plates securely to the rig.

Carefully remove the comb from the gel avoiding damaging the wells.

Using a wash bottle, carefully rinse each well with electrophoresis buffer.

Press plates into holder and fill the upper and lower reservoirs with electrophoresis buffer.

Using a bent syringe, blow out the bubbles from between the bottom of the plates.

7. Using a mortar and pestle grind plant sample in a suitable low salt buffer such as 10 mM phosphate, PBS or TE buffer, or in distilled water at a ratio of 1:20 (w/v). You may want to add a pinch of acid-washed sand to help in the homogenisation of the plant material. Centrifuge briefly to remove coarse plant material and place sample on ice.

Mix together in a fume hood one part clarified plant extract with one part 2 × SDS-PAGE sample buffer in an Eppendorf tube.

Close and secure lid before boiling for 10 minutes and place on ice immediately to cool to RT.

8. Add 1–15 µL samples to each well. The smaller the volume, the better the stacking and subsequent separation of proteins. Try to load similar volumes across the gel to avoid distortion of bands.

Attach the electrodes and hook gel rig up to power pack. Apply 100V constant voltage to run the sample through the stacking (upper) gel; once all of the sample has entered the separating (lower) gel, increase to 180V for the remainder of the electrophoresis until the bromophenol blue almost reaches the bottom of the gel.

Include prestained protein molecular weight markers as one of the samples. This will allow you to follow 1. separation of proteins during electrophoresis and subsequently 2. transfer from the gel to the membrane.

9. When the electrophoresis has finished, turn off the power and remove the electrodes.

Pour off the electrophoresis buffer.

Remove the gel plate assembly from the rig and place onto paper towels.

Carefully separate the plates with a spatula leaving the gel on one glass plate.

10. If the proteins in the gel are to be stained, at this point carefully lift the gel off the plate and transfer it into a container with Coomassie Blue stain and leave for 2 hours to overnight. Destain the gel by washing in several changes of destaining solution.

If the gel is to be electroblotted, at this point set up the blotting apparatus according to the instructions on page 72.

## **Western immunoblotting**

Wear gloves throughout this procedure. Do not let your bare skin come into contact with the nitrocellulose membrane as your skin will leave oil traces that will contribute to the background.

1. Cut a nitrocellulose (0.45 µm pore size) sheet to the size of the gel. For the Mini Protean II System, this is 8 × 10 cm.

Cut two pieces of Whatmann 3MM filter paper 8 × 10 cm.

Have 2–3 L of transfer buffer prepared and stored at 4°C.

Nylon membrane is generally not suitable for western blot detection and nitrocellulose has to be used.

2. Remove the gel from the glass plate and cut away the stacking gel and marker dye front. Mark the top left hand corner by cutting away a small piece of gel.

Transfer the gel to a plastic box containing transfer buffer and gently agitate for 10 minutes (10–15% gel) to 30 minutes (5–10% gel).

3. Have a second plastic box containing transfer buffer.

Carefully lower the nitrocellulose filter into the buffer, avoiding trapping air bubbles. Once it has wet through on the underside, gently submerge the filter in the buffer.

Also soak the filter paper and transfer sponges in the transfer buffer.

4. Prepare the sandwich for electroblotting by keeping everything submerged; this helps to avoid trapping air bubbles. Assemble on the cathode (the grey plastic support for the Mini Protean II System) in the following order: sponge-filter paper-gel-nitrocellulose-filter paper-sponge-clear plastic. Do this by placing a wet transfer sponge onto the cathode. Next, place a sheet of wet 3MM onto the sponge, removing air bubbles carefully. Place gel onto filter paper, removing air bubbles. Place the wet membrane onto the gel, placing it carefully into position over the gel. Rub the membrane with gloved fingers to remove air bubbles and to establish electrostatic interaction with gel. Place the other sheet of wet 3MM onto the filter, carefully removing air bubbles. Place other wet transfer sponge onto 3MM, removing air bubbles.

Close the sandwich (still submerged in transfer buffer), taking care not to slide the sandwich.

5. Place the electrophoresis tank onto a magnetic stirrer and add stirring bar.

Fill the transblot chamber with cold transfer buffer and an ice filled cooler, which will not leak into the buffer.

Slowly place assembly into the transblot chamber, the grey side facing the black electrode.

Hook up to power pack, ensuring the current direction is correct.

Electroblot at 100V (constant voltage) for 70 minutes.

Replace ice-filled cooler after 30 minutes.

6. Disassemble the apparatus and place membranes face-up on a piece of filter paper.

Air-dry and store at RT or proceed directly to detection steps.

Evaluate the success of the transfer by lack of bubble impressions on the membrane and by complete transfer of stained marker proteins. The gel can be stained with Commassie Blue as described above to assess extent of transfer.

## **Immuno detection**

1. Have ready a plastic box containing rinse buffer with 0.1% Triton X-100.  
Gently wet the membrane by placing it on top of the rinse buffer and once wet through underneath, gently submerging it.  
Shake gently for 10 minutes at RT.
2. Place membrane in 50 mL Blocking Solution (rinse buffer + 0.1% Triton X-100 + 5% (w/v) low fat (skim) milk powder)  
Incubate with gentle shaking for at least 1 hour at RT or overnight at 4°C.

The blocking solution should have a smooth consistency. Do not use it, if the milk powder will not fully dissolve or form clumps. If this happens, use a fresher batch of milk powder.

3. In as small a container as possible with membrane lying flat, place membrane face-up and add 10 mL of fresh rinse buffer containing 0.1% Triton X-100 + 1% skim milk powder + antiserum specific to the protein to be detected.  
Incubate with gentle agitation for 90 minutes at RT.

In most cases the antiserum used would have been generated in a rabbit. The dilution to be used will need to be determined empirically and depends on the specific antibody titre. A 1:10 000 dilution is a good place to start.

4. Pour off antibody solution.  
Wash vigorously in 4 changes of rinse buffer + 0.1% Triton X-100, 20 minutes each. Do not place more than one membrane per container, since membranes may stick to each other and prevent proper washes.
5. Add 10 mL of rinse buffer + 0.1% Triton X-100 + 1% skim milk powder + secondary goat anti-rabbit IgG-alkaline phosphatase conjugate. Incubate with gentle agitation for 90 minutes at RT or overnight at 4°C

For the dilution to use, follow the manufacturer's recommendation for immunoblot applications. Sigma Co. recommends 1:30 000 for its conjugate. If the source of your primary antibody is not from rabbit blood, you will need to use the appropriate antibody conjugate, which is specific for the species used.

6. Pour off antibody conjugate solution. Wash vigorously in 4 changes of rinse buffer + 0.1% Triton X-100, 20 minutes each. Do not place more than one membrane per container.
7. Equilibrate membrane by washing for 5 minutes in 10 mL of 1 × DIG detection buffer.
8. Dilute 10 µL of 10 mg/mL CPD Star luminescent substrate in 1 mL of 1 × DIG detection buffer.  
Stretch a large piece of plastic wrap onto the bench and tape down corners so that it is flat.  
Place membrane face-up on plastic wrap.  
Cover membrane with diluted CPD Star substrate solution. You will need about 1 mL per 25 cm<sup>2</sup> of membrane.  
Leave for 5 minutes at RT.

There are other sensitive chemiluminescent substrates for alkaline phosphatase or horseradish peroxidase. Your choice depends on what is available in your laboratory. Colourimetric substrates are generally not as sensitive.

9. Drain excess liquid from membrane and blot face-up briefly onto 3MM paper. Do not let the membrane dry out.

Place damp membrane between sheets of overhead transparency and expose to x-ray film. Refer to chapter 3.2 for notes regarding exposure to film.

## 3.5 Recipes

### Nucleic Acid Extraction

#### 4.4 M ammonium acetate

In a sterile bottle, dissolve 169.6 g of ammonium acetate in 400 mL of sterile distilled water.

Make up to 500 mL final volume.

Store at RT.

#### 4 M LiCl

Dissolve 17 g of lithium chloride in 100 mL of distilled water.

Add 0.1 mL diethyl pyrocarbonate (DEPC) and incubate for at least 12 hours at 37°C

Autoclave to sterilise.

Store at RT.

#### NTES

0.1 M NaCl.

10 mM Tris-HCl pH 8.0.

1 mM EDTA.

1.0% SDS.

Solutions containing SDS cannot be autoclaved as the detergent will foam. Therefore, make up the NTES mixture without the SDS, autoclave and add the SDS later. Ensure the SDS stock you use is RNase-free by making it up with DEPC-treated sterile distilled water. Alternatively, make the NTES up from sterile, DEPC-treated stock solutions and mix together in a sterile bottle.

#### Phenol/chloroform

The information provided here is from Sambrook et al. (1989). Phenol/chloroform is a 1:1 mixture of these two solutions. Phenol can come either as a clear, colourless liquid, which can be used without redistilling or in a crystalline form which does require redistilling. Sometimes, the liquefied form may be pink or yellow. If this occurs, do not use and return to the supplier for replacement. We recommend buying the liquefied form of phenol as it is simpler to work with. If you can only purchase the crystalline form, it must be redistilled at 160°C to remove oxidation products. If these are not removed, these products can cause cross-linking of RNA and DNA or cause the break down of the phosphodiester bonds of these molecules.

Phenol should be equilibrated to pH >7.8 otherwise the DNA will not partition into the aqueous phase. A method for the equilibration of phenol can be found in Sambrook et al. (1989).

Please note that phenol should be handled with extreme care in a fume hood and wearing protective clothing such as gloves, lab coat and safety glasses. It is a highly corrosive substance that can cause severe burns. If skin burns occur, wash with soap and water. Do not use ethanol to wash the area.

The phenol/chloroform mixture usually also contains isoamyl alcohol. The phenol denatures proteins, the chloroform debares proteins and starches and facilitates the separation of the phases and the isoamyl alcohol reduces foaming during the extraction. Only the phenol requires treatment prior to making up this mixture.

To make 500 mL of a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol mix together the following:

250 mL equilibrated phenol.

240 mL chloroform.

10 mL isoamyl alcohol.

Mix thoroughly.

Overlay with either 100 mM Tris-HCl pH 8.0 or TE pH 8.0.

Store in a dark bottle at 4°C for up to 1 month.

The shelf life can be extended with the addition of 8-hydroxyquinoline, which slows the oxidation process. Add a few crystals of this compound and dissolve by shaking. The organic phase will turn yellow, add enough crystals to turn the mixture canary yellow in colour.

## **10 mg/mL RNaseA**

RNase A can be purchased as a ready-made solution from most companies which sell molecular biology reagents. Ensure the RNase A is free of DNase. You can also buy it as a powder and make a stock solution as described below; however, it may be contaminated with DNase which must be removed by boiling.

Weigh out 100 mg RNase A into a sterile container.

Add 10 mL of sterile distilled water and dissolve the powder completely.

Place tube containing the solution into a boiling water bath for 10–15 minutes.

Aliquot into sterile Eppendorf tubes.

Store at –20°C.

While boiling the solution, ensure that the lid does not pop open, as any evaporation will change the concentration of the solution.

## **10% SDS**

Wearing a lab coat, gloves, safety glasses and a face mask, weigh out 50 g of SDS powder into a sterile bottle.

Add ~900 mL sterile distilled water carefully so as not to disturb the powder.

Once dissolved, make up to 1 L with sterile distilled water.

Store at RT.

Do not autoclave as this solution. At colder temperatures, the SDS may precipitate out of solution. If this occurs, heat until it has dissolved before you use it. For RNA work, ensure the solution is made with DEPC-treated sterile water. Use dedicated solutions for RNA work only.

### **TE (10 mM Tris-HCl/1 mM EDTA, pH 8.0)**

10 mL 1 M Tris-HCl pH 8.0.

2 mL 0.5 M EDTA pH 8.0.

988 mL distilled water.

Sterilise by autoclaving.

Store at RT.

For RNA work, make this solution using DEPC-treated water. Do not treat with DEPC afterwards.

### **TPS**

Using sterile stocks solutions, prepare 100 mL as follows in a sterile bottle:

10 mL 1 M Tris-HCl pH 8.0 (100 mM final concentration).

33.35 mL 3 M KCl (1 M final concentration).

2 mL 0.5 M EDTA pH 8.0 (10 mM final concentration).

54.67 mL sterile distilled water.

Store at RT.

### **Urea extraction buffer**

168 g urea.

25 mL 5 M NaCl.

20 mL 1 M Tris-HCl pH 8.0.

16 mL 0.5 M EDTA pH 8.0.

20 mL 20% sarcosine.

Make to 400 mL with sterile distilled water (~190 mL).

Store at RT.

Do not autoclave this solution as it contains urea. Instead, use autoclaved stocks and bottles.

### **0.05 M sodium phosphate buffer pH 8.0**

Combine 46.6 mL of 1 M  $\text{Na}_2\text{HPO}_4$  pH 8.0 and 3.4 mL  $\text{NaH}_2\text{PO}_4$  pH 8.0.

Make up to 1 L with distilled water and autoclave.

Store at RT.

## **DNA Restriction Digestion and Electrophoresis**

### **10 mg/mL ethidium bromide**

Ethidium bromide is a suspected mutagen. Where possible, avoid handling this compound in powder form to avoid inhaling it. When handling the liquid, always wear gloves. If possible, purchase pre-made ethidium bromide stock solution from a company selling molecular biology chemicals. If you must prepare the stock solution from powder, follow these instructions:

Ensure you are wearing a lab coat, safety glasses, gloves and a face mask.  
In a fume hood, weigh out 100 mg ethidium bromide powder into a sterile bottle.  
Carefully add 10 mL of sterile distilled water so as not to disperse the powder into the air.  
Add a magnetic stirring bar to the bottle, seal the bottle and cover with aluminium foil.  
Place on a magnetic stirrer overnight.  
Store at 4°C.

The magnetic stirring bar should be dedicated for use only with ethidium bromide.

### **6 × DNA loading dye**

40% sucrose (w/v) in sterile distilled water.

0.25% bromophenol blue.

Aliquot into 1 mL aliquots and store at 4°C.

(This and alternative dye solutions are described in Sambrook et al. (1989)).

### **0.1 M spermidine**

See the recipe section in chapter 2.3.

### **50 × TAE**

242 g Tris base.

57.1 mL glacial acetic acid.

100 mL 0.5 M EDTA pH 8.0.

Make up to 1L with distilled water and autoclave.

### **10 × TBE**

108g Tris base.

55 g boric acid.

40 mL 0.5 M EDTA pH 8.0.

Make up to 1L with distilled water and autoclave.

Alternatively, a 5 × stock can be made. For either concentration, a precipitate forms over time when concentrated TBE is stored. Try to store stocks in glass bottle at RT and discard batches that form the precipitate. While we have recommended a working strength of 1x, for agarose gels a 0.5 × solution will also provide enough buffering and current for electrophoresis.

### **10 × MOPS buffer**

0.2 M MOPS.

0.05 M sodium acetate.

0.01 M EDTA pH 8.0.

Adjust pH to 7.0 with glacial acetic acid.

Sterilise by autoclaving.

Store in the dark at RT (either in a darkened cupboard or cover the bottle in aluminium foil).

This solution will turn yellow on autoclaving.

## **RNA loading dye**

8% ficoll.  
0.02% bromophenol blue.  
0.04% xylene cyanol.  
Aliquot into 100 µL or smaller aliquots.  
Store aliquots at RT.

Discard each aliquot after use.

## **Protein Extraction**

### **30% Acrylamide/0.8% bisacrylamide (w/v)**

Purchase ready-made solution from a company.

### **4 × Upper Tris buffer**

6.06 g Tris-base (0.5 M Tris-HCl).  
Adjust pH to 6.8 with concentrated HCl.  
4 mL 10% SDS.  
Make to 100 mL with distilled water.  
Store at RT.

### **4 × Lower Tris buffer**

18.17 g Tris-base (1.5 M Tris-HCl).  
Adjust pH to 8.8.  
4 mL 10% SDS.  
Make to 100 mL with distilled water.

### **Tris-glycine electrophoresis buffer**

3 g Tris-base.  
14.3 g glycine.  
2 g SDS.  
Make to 1L with distilled water.  
Store at RT.

### **SDS-PAGE sample buffer**

10 mL glycerol.  
5 mL 2-mercaptoethanol.  
30 mL 10% SDS.  
12.5 mL 4 × Upper Tris buffer.  
Make up to 100 mL with distilled water.

### **Coomassie Blue staining solution**

Dissolve 0.1g Coomassie Blue R in 1 mL methanol.  
Add this to 200 mL 25% isopropanol/10% acetic acid.

## **Destaining solution**

10% isopropanol/10% acetic acid.

## **Southern/Northern Blotting and Hybridisation**

### **0.25 M HCl**

Add 21.55 mL of concentrated HCl into 800 mL of distilled water.

Make up to 1 L with distilled water.

Store at RT.

### **Denaturing solution (1.5 M NaOH/0.5 M NaCl)**

Dissolve 60 g NaOH and 29.22 g NaCl in ~800 mL of distilled water.

Make up to 1 L with distilled water.

Store at RT

### **Neutralising solution (1.5 M Tris-HCl pH 7.0/0.5 M NaCl)**

Dissolve 181.71 g Tris base and 29.22 g NaCl in ~800 mL of distilled water.

Add concentrated HCl until the pH reaches 7.0.

Make up to 1 L with distilled water.

Store at RT.

### **20 × SSC**

Dissolve 175.3g g of NaCl and 88.2 g of tri-sodium citrate in ~800 mL of distilled water.

Make up to 1 L with distilled water.

Sterilise by autoclaving.

Store at RT.

For RNA work, treat this solution with DEPC prior to autoclaving and use only for RNA work.

### **10 mg/mL herring/salmon sperm DNA**

Weigh out 200 mg of herring or salmon sperm DNA.

Add ~12 mL of distilled water.

Dissolve the DNA by stirring and heating.

Make up to 20 mL with distilled water.

Denature the DNA by sucking up and squeezing out through a 21 gauge needle.

Further denature by autoclaving.

Aliquot into 0.5 mL aliquots in sterile Eppendorf tubes.

Store at -20°C.

This solution is quite viscous before autoclaving. It is difficult to dissolve the DNA without heating. Take care not to exceed the total volume of 20 mL since the DNA itself takes up a large volume.

## **Hybridisation solution for radioactively labelled probes**

0.9 M NaCl.

1% NaCl.

10% dextran sulphate.

100 µg/mL herring or salmon sperm DNA.

To make up 100 mL of this solution, using sterile solutions where appropriate, combine together.

18 mL 5 M NaCl.

10 mL 10% SDS.

10 g dextran sulphate.

Add ~20 mL sterile distilled water.

Stir and heat to dissolve the dextran sulphate.

Slowly add the remaining volume of water (52 mL) to make up to 100 mL, ensuring the dextran sulphate is dissolved completely.

Store at RT.

This solution is fairly viscous. The dextran sulphate is difficult to dissolve and takes up a large volume, thus you need to be careful that you don't add all of the water in one go as the final volume will be greater than 100 mL once the dextran sulphate has dissolved. On storage, some of the dextran sulphate comes out of solution so that just prior to use, heat for 1–2 minutes in a microwave or over a Bunsen burner until it is completely dissolved.

Make up this mixture without the herring sperm DNA and store at RT. Add the herring or salmon sperm DNA just prior to hybridisation as described in the procedure outline.

## **DIG Easy Hyb Solution**

This particular solution must be purchased from Roche Diagnostics (formerly Boehringer Mannheim). The recipe for this solution is a trade secret and therefore cannot be reproduced in the laboratory.

Northern hybridisation can be carried out using DIG labelled probes, although the sensitivity is not as great as with radioactively labelled probes. If you wish to use a DIG-labelled probe for northern, use a fresh bottle of DIG Easy Hyb and keep it dedicated for RNA work.

## **2 × SSC/0.1% SDS**

Mix together 100 mL 20 × SSC and 10 mL 10% SDS.

Add 890 mL sterile distilled water.

Store at RT.

Refer to the notes for 10% SDS.

## **0.2 × SSC /0.1% SDS**

Mix together 10 mL 20 × SSC and 10 mL 10% SDS.

Add 980 mL sterile distilled water.

Store at RT.

Refer to the notes for 10% SDS.

## **10% Blocking solution**

Blocking reagent must be purchased from Roche Diagnostics. As for DIG Easy Hyb, the recipe for this reagent is a trade secret and therefore can't be reproduced in the laboratory.

Aliquot blocking reagent upon opening into ~50 mL aliquots, using sterile 50 mL tubes or bottles.

Prior to aliquoting, store at RT. After opening and aliquoting, store at -20°C.

If using DIG-labelled probes for RNA work, keep an aliquot dedicated for this.

For immediate use as 2 × solution mix:

20 mL Blocking Reagent.

80 mL 1 × maleic acid buffer.

## **10 × Maleic acid buffer**

This buffer can be purchased from Roche Diagnosits. However, the recipe for this is available and can thus be made up in the laboratory as follows:

To 800 mL distilled water add:

116 g maleic acid.

87.6 g NaCl.

70 g NaOH beads.

Adjust pH to 7.5 with 8 N NaOH.

Fill up to 1 L with distilled water.

To make a 1 × working solution, dilute the stock 10×. For RNA work, ensure the solution is RNase-free.

## **10 × DIG washing buffer**

This buffer can be purchased from Roche Diagnosits. However, the recipe for this is available and can thus be made up in the laboratory as follows:

Add 30 mL Tween 20 to 1 L 10 × maleic acid buffer.

To make a 1 × working solution, dilute the stock 10×. For RNA work, ensure the solution is RNase-free.

## **10 × DIG detection buffer**

This buffer can be purchased from Roche Diagnosits. However, the recipe for this is available and can thus be made up in the laboratory as follows:

121.14 g Tris.

58.44 g NaCl.

Fill up to 1 L with distilled water.

Adjust pH to 9.5.

To make a 1 × working solution, dilute the stock 10×. For RNA work, ensure the solution is RNase-free.

## **ELISA buffers**

All the buffers described below are as described by Clark and Adams (1977).

### **10 × Phosphate buffered saline (PBS) pH 7.4**

80 g/L NaCl.

2 g/L KH<sub>2</sub>PO<sub>4</sub>.

11.5 g/L Na<sub>2</sub>HPO<sub>4</sub>.

2 g/L KCl.

Adjust pH to 7.4.

Sterilise by autoclaving.

Store at RT.

To make a 1 × working solution, dilute the stock 10×.

### **PBS-Tween**

To 1 L of 1 × PBS pH 7.4, add 0.5 mL of Tween-20.

Store in wash bottles at 4°C.

### **Sample buffer**

In a sterile bottle, add 2 g of soluble polyvinyl pyrrolidone (PVP) to 100 mL of fresh PBS-Tween.

Adjust pH to 6.0 and store at 4°C.

### **Conjugate buffer**

In a sterile bottle, add 2 g PVP and 0.2 g bovine serum albumin (BSA) to 100 mL of fresh PBS-Tween.

Store at 4°C.

### **Coating buffer**

In a sterile bottle, dissolve 0.159 g of Na<sub>2</sub>CO<sub>3</sub> and 0.293 g NaHCO<sub>3</sub> in 70 mL of sterile distilled water.

Adjust pH to 9.6 with glacial acetic acid.

Make up to 100 mL with sterile distilled water and store at 4°C.

### **Substrate buffer**

In a sterile bottle, mix 9.7 mL diethanolamine with 80 mL sterile distilled water.

Adjust pH to 9.8 with HCl.

Make up to 100 mL with sterile distilled water and store at 4°C.

## **Immuno (Western) Blotting**

### **Protein Transfer buffer**

14.4 g/L glycine (192 mM).

3.03 g/L Tris base (25 mM).

200 mL/L ethanol (20%).

Add distilled water to 1 L; do not adjust pH.

Store at 4°C.

### **10 × Rinse buffer**

12.1 g/L Tris-HCl pH 7.4 (10 mM).

87.6 g/L NaCl (150 mM).

3.7 g/L EDTA (1 mM).

Add distilled water to 1 L.

Store at 4°C.

## REFERENCES

- Atreya, C.D., Rao, J.P. and Subrahmanyam, N.C. 1984. In vitro regeneration of peanut (*Arachis hypogaea* L.) plantlets from embryo axes and cotyledon segments. *Plant Science Letters*, 34: 379–83.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1997. Identification of newly transcribed RNA. In: *Current Protocols in Molecular Biology*. John Wiley and Sons Inc, USA, 4–10.
- Baker, C.M. and Wetzstein, H.Y. 1992. Somatic embryogenesis and plant regeneration from leaflets of peanut, *Arachis hypogaea*. *Plant Cell Reports*, 11: 71–75.
- Baker, C.M. and Wetzstein, H.Y. 1998. Leaflet development, induction time, and medium influence somatic embryogenesis in peanut (*Arachis hypogaea* L.). *Plant Cell Reports*, 17: 925–29.
- Baker, C.M., Durham, R.E., Burns, J.A., Parrott, W.A. and Wetzstein, H.Y. 1995. High frequency somatic embryogenesis in peanut (*Arachis hypogaea* L.) using mature, dry seed. *Plant Cell Reports*, 15: 38–42.
- Brar, G.S., Cohen, B.A., Vick, C.A. and Johnson, G.W. 1994. Recovery of transgenic peanut (*Arachis hypogaea* L.) plants from elite cultivars utilising ACCELL technology. *The Plant Journal*, 5: 745–53.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. *Science*, 263: 802–805.
- Cheng, M., His, D.C.H. and Phillips, G.C. 1994. Recovery of primary transformants of Valencia-type peanut using *Agrobacterium tumefaciens*. *Peanut Science*, 21: 84–88.
- Cheng, M., Jarret, R.L., Li, Z. and Demski, J.W. 1997. Expression and inheritance of foreign genes in transgenic peanut plants generated by *Agrobacterium*-mediated transformation. *Plant Cell Reports*, 16: 541–544.
- Cheng, M., Jarret, R.L., Li, Z., Xing, A. and Demski, J.W. 1996. Production of fertile transgenic peanut (*Arachis hypogaea* L.) plants using *Agrobacterium tumefaciens*. *Plant Cell Reports*, 15: 653–657.
- Chilton, M.D. 1983. Crown gall gene as a vector for plants. *Bio/technology*, 1: 163–164.
- Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, 34: 475–483.
- Clemente, T.E., Robertson, D., Islieb, T.G. and Beute-Marvin, K. 1992. Evaluation of peanut (*Arachis hypogaea* L.) leaflets form mature zygotic embryos as recipient tissue for biolistic gene transfer. *Transgenic Research*, 1: 275–284.
- De Freitas, V.G., Lacrote, C., Sachette, M.G., Krul, W.R., De Oliveira, D.E., Neves, L.J. and Mansur, E. 1997. Identification of competent cells for *Agrobacterium*-transformation and in vitro regeneration in peanut leaf and cotyledon explants. *Revista Brasileira de Fisiologia Vegetal*, 9: 157–167.
- Dewi, I.S., Harjosudarmo, J., Birch, R.G. and Dietzgen, R.G. 1997. The effect of 2,4-D, NAA, and picloram on somatic embryogenesis and plant regeneration form immature seed. *Jurnal Bioteknologi Pertanian*, 2: 23–30.
- Dietzgen, R.G. (ed.) 1999. Elimination of aflatoxin contamination in peanut. Australian Centre for International Agricultural Research, Canberra, ACT. Proceedings No. 89.

- Dietzgen R.G. 2000. Applications of PCR in plant virology. In: Khan, J.A. and Dijkstra, J. (ed.). Trends in Plant Virology. Harworth Press, New York.
- Eapen, S. and George, L. 1993. Somatic embryogenesis in peanut: Influence of growth regulators and sugars. *Plant Cell, Tissue and Organ Culture*, 35: 151–156.
- Eapen, S. and George, L. 1994. *Agrobacterium tumefaciens* mediated gene transfer in peanut (*Arachis hypogaea* L.) *Plant Cell Reports*, 13: 582–586.
- Egnin, M., Mora, A. and Prakash, C. 1998. Factors enhancing *Agrobacterium tumefaciens*-mediated gene transfer in peanut (*Arachis hypogaea* L.). *In Vitro Cellular and Developmental Plant Biology*, 34: 310–318.
- Fang, X., Xu, Z., Zhang, Z., Yan, L., Chen, K., Jia, S., Zhang, C., Livingstone, M.D., Birch, R.G. and Dietzgen, R.G. 1996. Plant regeneration and *Agrobacterium*-mediated gene transfer in leaflets of peanut (*Arachis hypogaea* L.). *Oil Crops of China*, 4: 52–56.
- Fang, X., Xu, Z., Zhang, Z., Yan, L., Chen, K., Luo, L., Chen, J., Birch, R.G. and Dietzgen, R.G. 1999. Expression and inheritance of GUS gene and NPTII gene in transgenic peanut plants generated by *Agrobacterium*-mediated transformation. *Peanut Science and Technology*, 35: 241–245.
- Finer, J.J., Vain, P., Jones, M.W. and McMullen, M.D. 1992. Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Reports*, 11: 323–328.
- Franklin, C.I., Shorosh, K.M., Trieu, A.N., Cassidy, B.G. and Nelson, R.S. 1993. Stable transformation of peanut callus via *Agrobacterium* — mediated DNA transfer. *Transgenic Research*, 2: 321–324.
- Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter*, 5: 387–405.
- Hazra, S., Sathaye, S. and Mascarenhas, A.F. 1989. Direct somatic embryogenesis in peanut (*Arachis hypogaea* L.). *Bio/technology*, 7: 949–951.
- Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant Journal*, 6: 271–282.
- Hood, E.E., Helmer, G.L., Frayley, R.T. and Chilton, M.D. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *Journal of Bacteriology*, 168: 1291–1301.
- Khan, M.R.I., Tabe, L.M., Heath, L.C., Spencer, D. and Higgins, T.J.V. 1994. *Agrobacterium* — mediated transformation of subterranean clover (*Trifolium subterraneum* L.). *Plant Physiology*, 105: 81–88.
- Kochert, G. 1996. Molecular markers and genome mapping. In: Darussamin, A. (ed.) *Current Status of Agricultural Biotechnology in Indoensia*, 89–108. AARD, Jakarta.
- Kokalis-Burelle, N., Porter, D.M., Rodriguez-Kabana, R., Smith, D.H. and Subrahmanyam, P. (ed.) 1997. *Compendium of Peanut Diseases*, Secondnd Edition. St. Paul, USA: APS Press.
- Lacorte, C., Mansur, E., Timmerman, B. and Cordeiro, A.R. 1991. Gene transfer into peanut (*Arachis hypogaea* L.) by *Agrobacterium tumefaciens*. *Plant Cell Reports*, 10: 354–357.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, 227: 680–685.
- Li, Z., Jarret, R.L., Pittman, R.N., Dunbar, K.B. and Demski, J.W. 1993. Efficient plant regeneration from protoplasts of *Arachis paraguariensis* Chod. Et Hassl. using a nurse culture method. *Plant Cell, Tissue and Organ Culture*, 34: 83–90.

- Livingstone, D.M. and Birch, R.G. 1995. Plant regeneration and microprojectile-mediated gene transfer in embryonic leaflets of peanut (*Arachis hypogaea* L.). Australian Journal of Plant Physiology, 22: 585–591.
- Livingstone, D.M. and Birch, R.G. 1999. Efficient transformation and regeneration of diverse cultivars of peanut (*Arachis hypogaea* L.) by particle bombardment into embryogenic callus produced from mature seeds. Molecular Breeding, 5: 43–51.
- Mansur, E.A., Lacorte, C., De Freitas, V.G., De Oliveira, D.E., Timmerman, B. and Vordeiro, A.R. 1993. Regulation of transformation efficiency of peanut (*Arachis hypogaea* L.) explants by *Agrobacterium tumefaciens*. Plant Science, 89: 93–99.
- McFarlane, M. and Wilson, J.B. 1996. A model for the mechanism for precise integration of a microinjected transgene. Transgenic Research, 5: 171–177.
- McKently, A.H., Moore, G.A., Doostdar, H., Niedz, R.P. 1995. *Agrobacterium*-mediated transformation of peanut (*Arachis hypogaea* L.) embryo axes and the development of transgenic plants. Plant Cell Reports, 14: 699–703.
- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473–497.
- Newton, T.R. 1997. *Agrobacterium*-mediated transformation of peanut (*Arachis hypogaea* L.) Honours Thesis, The University of Queensland.
- Ozias-Akins, P. 1989. Plant regeneration from immature embryos of peanut. Plant Cell Reports, 8: 217–218.
- Ozias-Akins, P., Fan, H. and Wang, A. 1996. Expression of GUS under the control of a soybean promoter modulated by carbohydrates. Proceedings of the American Peanut Research and Education Society Inc., Page 27.
- Ozias-Akins, P., Schnall, J.A., Anderson, W.F., Singsit, C., Clemente, T.E., Adang, M.J. and Weissinger, A.K. 1993. Regeneration of transgenic peanut plants from stably transformed embryogenic callus. Plant Science, 93: 185–194.
- Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular Cloning, a Laboratory Manual, Second Edition. Cold Spring Harbour Laboratory Press, New York.
- Sarker, R.H., Chowdury, A.P. and Hoque, M.I. 1997. Preliminary studies on *Agrobacterium*-mediated genetic transformation of peanut (*Arachis hypogaea* L.) Bangaldesh Journal of Botany, 26: 155–162.
- Smith, N. 1998. More T-DNA than meets the eye. Trends in Plant Science, 3: 85.
- Smith, R.L. and Beliaev, D.V. 1995 Isolation of peanut seed coat- and pod-specific genes using differential hybridization and differential display methods. Proceedings of the American Peanut Research and Education Society Inc., 27.
- Smith, G.R., van de Velde, R. and Dale, J.L. 1992. PCR amplification of a specific double-stranded RNA region of Fiji disease virus from diseased sugarcane. Journal of Virological Methods, 39: 237–246.
- Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology, 98: 503–517.
- Urban, L.A. and Weissinger, A.K. 1996. Enhanced regeneration and transformation of Valencia A peanut with the osmotin gene. Proceedings of The American Peanut Research and Education Society Inc., Page 27.
- Yang, H., Singsit, C., Wang, A., Gonsalves, D. and Ozias-Akins, P. 1998. Transgenic peanut plants containing a nucleocapsid protein gene of tomato spotted wilt virus show divergent levels of gene expression. Plant Cell Reports, 17: 693–699.